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(54) Title: GENETIC INHIBITION BY DOUBLE-STRANDED RNA

(57) Abstract

A process is provided of introducing an RNA into a living cell to inhibit gene expression of a target gene in that cell. The process may be practiced *ex vivo* or *in vivo*. The RNA has a region with double-stranded structure. Inhibition is sequence-specific in that the nucleotide sequences of the duplex region of the RNA and of a portion of the target gene are identical. The present invention is distinguished from prior art interference in gene expression by antisense or triple-strand methods.

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GENETIC INHIBITION BY DOUBLE-STRANDED RNA

GOVERNMENT RIGHTS

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BACKGROUND OF THE INVENTION

1. Field of the Invention

The present invention relates to gene-specific inhibition of gene expression by double-stranded ribonucleic acid (dsRNA).

2. Description of the Related Art

Targeted inhibition of gene expression has been a long-felt need in biotechnology and genetic engineering. Although a major investment of effort has been made to achieve this goal, a more comprehensive solution to this problem was still needed.

Classical genetic techniques have been used to isolate mutant organisms with reduced expression of selected genes. Although valuable, such techniques require laborious mutagenesis and screening programs, are limited to organisms in which genetic manipulation is well established (e.g., the existence of selectable markers, the ability to control genetic segregation and sexual reproduction), and are limited to applications in which a large number of cells or organisms can be sacrificed to isolate the desired mutation. Even under these circumstances, classical genetic techniques can fail to produce mutations in specific target genes of interest, particularly when complex genetic pathways are involved. Many applications of molecular genetics require the ability to go beyond classical genetic screening techniques and efficiently produce a *directed* change in gene expression in a specified group of cells or organisms. Some such applications are knowledge-based projects in which it is of importance to understand what effects the loss of a specific gene product (or products) will have on the behavior of the cell or organism. Other applications are engineering based, for example: cases in which is important to

produce a population of cells or organisms in which a specific gene product (or products) has been reduced or removed. A further class of applications is therapeutically based in which it would be valuable for a functioning organism (e.g., a human) to reduce or remove the amount of a specified gene product (or products). Another class of applications provides a disease model in which a physiological function in a living organism is genetically manipulated to reduce or remove a specific gene product (or products) without making a permanent change in the organism's genome.

In the last few years, advances in nucleic acid chemistry and gene transfer have inspired new approaches to engineer specific interference with gene expression. These approaches are described below.

Use of Antisense Nucleic Acids to Engineer Interference

Antisense technology has been the most commonly described approach in protocols to achieve gene-specific interference. For antisense strategies, stochiometric amounts of single-stranded nucleic acid complementary to the messenger RNA for the gene of interest are introduced into the cell. Some difficulties with antisense-based approaches relate to delivery, stability, and dose requirements. In general, cells do not have an uptake mechanism for single-stranded nucleic acids, hence uptake of unmodified single-stranded material is extremely inefficient. While waiting for uptake into cells, the single-stranded material is subject to degradation. Because antisense interference requires that the interfering material accumulate at a relatively high concentration (at or above the concentration of endogenous mRNA), the amount required to be delivered is a major constraint on efficacy. As a consequence, much of the effort in developing antisense technology has been focused on the production of modified nucleic acids that are both stable to nuclease digestion and able to diffuse readily into cells. The use of antisense interference for gene therapy or other whole-organism applications has been limited by the large amounts of oligonucleotide that need to be synthesized from non-natural analogs, the cost of such synthesis, and the difficulty even with high doses of maintaining a sufficiently concentrated and uniform pool of interfering material in each cell.

Triple-Helix Approaches to Engineer Interference

A second, proposed method for engineered interference is based on a triple helical nucleic acid structure. This approach relies on the rare ability of certain nucleic acid populations to adopt a triple-stranded structure. Under physiological conditions, nucleic acids are virtually all single- or double-stranded, and rarely if ever form triple-stranded structures. It has been known for some time, however, that certain simple purine- or pyrimidine-rich sequences could form a triple-stranded molecule *in vitro* under extreme conditions of pH (i.e., in a test tube). Such structures are generally very transient under physiological conditions, so that simple delivery of unmodified nucleic acids designed to produce triple-strand structures does not yield interference. As with antisense, development of triple-strand technology for use *in vivo* has focused on the development of modified nucleic acids that would be more stable and more readily absorbed by cells *in vivo*. An additional goal in developing this technology has been to produce modified nucleic acids for which the formation of triple-stranded material proceeds effectively at physiological pH.

Co-Suppression Phenomena and Their Use in Genetic Engineering

A third approach to gene-specific interference is a set of operational procedures grouped under the name "co-suppression". This approach was first described in plants and refers to the ability of transgenes to cause silencing of an unlinked but homologous gene. More recently, phenomena similar to co-suppression have been reported in two animals: *C. elegans* and *Drosophila*. Co-suppression was first observed by accident, with reports coming from groups using transgenes in attempts to achieve over-expression of a potentially useful locus. In some cases the over-expression was successful while, in many others, the result was opposite from that expected. In those cases, the transgenic plants actually showed less expression of the endogenous gene. Several mechanisms have so far been proposed for transgene-mediated co-suppression in plants; all of these mechanistic proposals remain hypothetical, and no definitive mechanistic description of the process has been presented. The models that have been proposed to explain co-suppression can be placed in two different categories. In one set of proposals, a direct physical interaction at the DNA- or chromatin-level between two different chromosomal sites has been

hypothesized to occur; an as-yet-unidentified mechanism would then lead to *de novo* methylation and subsequent suppression of gene expression. Alternatively, some have postulated an RNA intermediate, synthesized at the transgene locus, which might then act to produce interference with the endogenous gene. The characteristics of the interfering RNA, as well as the nature of the interference process, have not been determined. Recently, a set of experiments with RNA viruses have provided some support for the possibility of RNA intermediates in the interference process. In these experiments, a replicating RNA virus is modified to include a segment from a gene of interest. This modified virus is then tested for its ability to interfere with expression of the endogenous gene. Initial results with this technique have been encouraging, however, the properties of the viral RNA that are responsible for interference effects have not been determined and, in any case, would be limited to plants which are hosts of the plant virus.

Distinction between the Present Invention and Antisense Approaches

The present invention differs from antisense-mediated interference in both approach and effectiveness. Antisense-mediated genetic interference methods have a major challenge: delivery to the cell interior of specific single-stranded nucleic acid molecules at a concentration that is equal to or greater than the concentration of endogenous mRNA. Double-stranded RNA-mediated inhibition has advantages both in the stability of the material to be delivered and the concentration required for effective inhibition. Below, we disclose that in the model organism *C. elegans*, the present invention is at least 100-fold more effective than an equivalent antisense approach (i.e., dsRNA is at least 100-fold more effective than the injection of purified antisense RNA in reducing gene expression). These comparisons also demonstrate that inhibition by double-stranded RNA must occur by a mechanism distinct from antisense interference.

Distinction between the Present Invention and Triple-Helix Approaches

The limited data on triple strand formation argues against the involvement of a stable triple-strand intermediate in the present invention. Triple-strand structures occur rarely, if at all, under physiological conditions and are limited to very unusual base sequence with long runs of purines and pyrimidines. By contrast, dsRNA-mediated

inhibition occurs efficiently under physiological conditions, and occurs with a wide variety of inhibitory and target nucleotide sequences. The present invention has been used to inhibit expression of 18 different genes, providing phenocopies of null mutations in these genes of known function. The extreme environmental and sequence constraints on triple-helix formation make it unlikely that dsRNA-mediated inhibition *in C. elegans* is mediated by a triple-strand structure.

Distinction between Present Invention and Co-Suppression Approaches

The transgene-mediated genetic interference phenomenon called co-suppression may include a wide variety of different processes. From the viewpoint of application to other types of organisms, the co-suppression phenomenon in plants is difficult to extend. A confounding aspect in creating a general technique based on co-suppression is that some transgenes in plants lead to suppression of the endogenous locus and some do not. Results in *C. elegans* and *Drosophila* indicate that certain transgenes can cause interference (i.e., a quantitative decrease in the activity of the corresponding endogenous locus) but that most transgenes do not produce such an effect. The lack of a predictable effect in plants, nematodes, and insects greatly limits the usefulness of simply adding transgenes to the genome to interfere with gene expression. Viral-mediated co-suppression in plants appears to be quite effective, but has a number of drawbacks. First, it is not clear what aspects of the viral structure are critical for the observed interference. Extension to another system would require discovery of a virus in that system which would have these properties, and such a library of useful viral agents are not available for many organisms. Second, the use of a replicating virus within an organism to effect genetic changes (e.g., long- or short-term gene therapy) requires considerably more monitoring and oversight for deleterious effects than the use of a defined nucleic acid as in the present invention.

The present invention avoids the disadvantages of the previously-described methods for genetic interference. Several advantages of the present invention are discussed below, but numerous others will be apparent to one of ordinary skill in the biotechnology and genetic engineering arts.

SUMMARY OF THE INVENTION

A process is provided for inhibiting expression of a target gene in a cell. The process comprises introduction of RNA with partial or fully double-stranded character into the cell or into the extracellular environment. Inhibition is specific in that a nucleotide sequence from a portion of the target gene is chosen to produce inhibitory RNA. We disclose that this process is (1) effective in producing inhibition of gene expression, (2) specific to the targeted gene, and (3) general in allowing inhibition of many different types of target gene.

The target gene may be a gene derived from the cell, an endogenous gene, a transgene, or a gene of a pathogen which is present in the cell after infection thereof. Depending on the particular target gene and the dose of double stranded RNA material delivered, the procedure may provide partial or complete loss of function for the target gene. A reduction or loss of gene expression in at least 99% of targeted cells has been shown. Lower doses of injected material and longer times after administration of dsRNA may result in inhibition in a smaller fraction of cells. Quantitation of gene expression in a cell may show similar amounts of inhibition at the level of accumulation of target mRNA or translation of target protein.

The RNA may comprise one or more strands of polymerized ribonucleotide; it may include modifications to either the phosphate-sugar backbone or the nucleoside. The double-stranded structure may be formed by a single self-complementary RNA strand or two complementary RNA strands. RNA duplex formation may be initiated either inside or outside the cell. The RNA may be introduced in an amount which allows delivery of at least one copy per cell. Higher doses of double-stranded material may yield more effective inhibition. Inhibition is sequence-specific in that nucleotide sequences corresponding to the duplex region of the RNA are targeted for genetic inhibition. RNA containing a nucleotide sequences identical to a portion of the target gene is preferred for inhibition. RNA sequences with insertions, deletions, and single point mutations relative to the target sequence have also been found to be effective for inhibition. Thus, sequence identity may optimized by alignment algorithms known in the art and calculating the percent difference between the nucleotide sequences. Alternatively, the duplex region of the RNA may be

defined functionally as a nucleotide sequence that is capable of hybridizing with a portion of the target gene transcript.

The cell with the target gene may be derived from or contained in any organism (e.g., plant, animal, protozoan, virus, bacterium, or fungus). RNA may be synthesized either *in vivo* or *in vitro*. Endogenous RNA polymerase of the cell may mediate transcription *in vivo*, or cloned RNA polymerase can be used for transcription *in vivo* or in *vitro*. For transcription from a transgene *in vivo* or an expression construct, a regulatory region may be used to transcribe the RNA strand (or strands).

The RNA may be directly introduced into the cell (i.e., intracellularly); or introduced extracellularly into a cavity, interstitial space, into the circulation of an organism, introduced orally, or may be introduced by bathing an organism in a solution containing RNA. Methods for oral introduction include direct mixing of RNA with food of the organism, as well as engineered approaches in which a species that is used as food is engineered to express an RNA, then fed to the organism to be affected. Physical methods of introducing nucleic acids include injection directly into the cell or extracellular injection into the organism of an RNA solution.

The advantages of the present invention include: the ease of introducing double-stranded RNA into cells, the low concentration of RNA which can be used, the stability of double-stranded RNA, and the effectiveness of the inhibition. The ability to use a low concentration of a naturally-occurring nucleic acid avoids several disadvantages of antisense interference. This invention is not limited to *in vitro* use or to specific sequence compositions, as are techniques based on triple-strand formation. And unlike antisense interference, triple-strand interference, and co-suppression, this invention does not suffer from being limited to a particular set of target genes, a particular portion of the target gene's nucleotide sequence, or a particular transgene or viral delivery method. These concerns have been a serious obstacle to designing general strategies according to the prior art for inhibiting gene expression of a target gene of interest.

Furthermore, genetic manipulation becomes possible in organisms that are not classical genetic models. Breeding and screening programs may be accelerated by the ability to rapidly assay the consequences of a specific, targeted gene disruption. Gene disruptions may be used to discover the function of the target gene, to produce disease

models in which the target gene are involved in causing or preventing a pathological condition, and to produce organisms with improved economic properties.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 shows the genes used to study RNA-mediated genetic inhibition in *C. elegans*. Intron-exon structure for genes used to test RNA-mediated inhibition are shown (exons: filled boxes; introns: open boxes; 5' and 3' untranslated regions: shaded; *unc-22* [9], *unc-54* [12], *fem-1* [14], and *hlh-1* [15]).

Figures 2 A-I show analysis of inhibitory RNA effects in individual cells. These experiments were carried out in a reporter strain (called PD4251) expressing two different reporter proteins, nuclear GFP-LacZ and mitochondrial GFP. The micrographs show progeny of injected animals visualized by a fluorescence microscope. Panels A (young larva), B (adult), and C (adult body wall; high magnification) result from injection of a control RNA (ds-*unc22A*). Panels D-F show progeny of animals injected with ds-*gfpG*. Panels G-I demonstrate specificity. Animals are injected with ds-*lacZL* RNA, which should affect the nuclear but not the mitochondrial reporter construct. Panel H shows a typical adult, with nuclear GFP-LacZ lacking in almost all body-wall muscles but retained in vulval muscles. Scale bars are 20 μm.

Figures 3 A-D show effects of double-stranded RNA corresponding to *mex-3* on levels of the endogenous mRNA. Micrographs show *in situ* hybridization to embryos (dark stain). Panel A: Negative control showing lack of staining in the absence of hybridization probe. Panel B: Embryo from uninjected parent (normal pattern of endogenous *mex-3* RNA[20]). Panel C: Embryo from a parent injected with purified *mex-3*B antisense RNA. These embryos and the parent animals retain the *mex-3* mRNA, although levels may have been somewhat less than wild type. Panel D: Embryo from a parent injected with dsRNA corresponding to *mex-3*B; no *mex-3* RNA was detected. Scale: each embryo is approximately 50 μm in length.

Figure 4 shows inhibitory activity of *unc-22A* as a function of structure and concentration. The main graph indicates fractions in each behavioral class. Embryos in the uterus and already covered with an eggshell at the time of injection were not affected and, thus, are not included. Progeny cohort groups are labeled **1** for 0-6 hours, **2** for 6-15

hours, 3 for 15-27 hours, 4 for 27-41 hours, and 5 for 41-56 hours The bottom-left diagram shows genetically derived relationship between *unc-22* gene dosage and behavior based on analyses of *unc-22* heterozygotes and polyploids[8,3].

Figures 5 A-C show examples of genetic inhibition following ingestion by *C. elegans* of dsRNAs from expressing bacteria. Panel A: General strategy for production of dsRNA by cloning a segment of interest between flanking copies of the bacteriophage T7 promoter and transcribing both strands of the segment by transfecting a bacterial strain (BL21/DE3)[28] expressing the T7 polymerase gene from an inducible (Lac) promoter. Panel B: A GFP-expressing *C. elegans* strain, PD4251 (see Figure 2), fed on a native bacterial host. Panel C: PD4251 animals reared on a diet of bacteria expressing dsRNA corresponding to the coding region for *gfp*.

DETAILED DESCRIPTION OF THE INVENTION

The present invention provides a method of producing sequence-specific inhibition of gene expression by introducing double-stranded RNA (dsRNA). A process is provided for inhibiting expression of a target gene in a cell. The process comprises introduction of RNA with partial or fully double-stranded character into the cell. Inhibition is sequence-specific in that a nucleotide sequence from a portion of the target gene is chosen to produce inhibitory RNA. We disclose that this process is (1) effective in producing inhibition of gene expression, (2) specific to the targeted gene, and (3) general in allowing inhibition of many different types of target gene.

The target gene may be a gene derived from the cell (i.e., a cellular gene), an endogenous gene (i.e., a cellular gene present in the genome), a transgene (i.e., a gene construct inserted at an ectopic site in the genome of the cell), or a gene from a pathogen which is capable of infecting an organism from which the cell is derived. Depending on the particular target gene and the dose of double stranded RNA material delivered, this process may provide partial or complete loss of function for the target gene. A reduction or loss of gene expression in at least 99% of targeted cells has been shown.

Inhibition of gene expression refers to the absence (or observable decrease) in the level of protein and/or mRNA product from a target gene. Specificity refers to the ability to inhibit the target gene without manifest effects on other genes of the cell. The

consequences of inhibition can be confirmed by examination of the outward properties of the cell or organism (as presented below in the examples) or by biochemical techniques such as RNA solution hybridization, nuclease protection, Northern hybridization, reverse transcription, gene expression monitoring with a microarray, antibody binding, enzyme linked immunosorbent assay (ELISA), Western blotting, radioimmunoassay (RIA), other immunoassays, and fluorescence activated cell analysis (FACS). For RNA-mediated inhibition in a cell line or whole organism, gene expression is conveniently assayed by use of a reporter or drug resistance gene whose protein product is easily assayed. Such reporter genes include acetohydroxyacid synthase (AHAS), alkaline phosphatase (AP), beta galactosidase (LacZ), beta glucoronidase (GUS), chloramphenicol acetyltransferase (CAT), green fluorescent protein (GFP), horseradish peroxidase (HRP), luciferase (Luc), nopaline synthase (NOS), octopine synthase (OCS), and derivatives thereof. Multiple selectable markers are available that confer resistance to ampicillin, bleomycin, chloramphenicol, gentamycin, hygromycin, kanamycin, lincomycin, methotrexate, phosphinothricin, puromycin, and tetracyclin.

Depending on the assay, quantitation of the amount of gene expression allows one to determine a degree of inhibition which is greater than 10%, 33%, 50%, 90%, 95% or 99% as compared to a cell not treated according to the present invention. Lower doses of injected material and longer times after administration of dsRNA may result in inhibition in a smaller fraction of cells (e.g., at least 10%, 20%, 50%, 75%, 90%, or 95% of targeted cells). Quantitation of gene expression in a cell may show similar amounts of inhibition at the level of accumulation of target mRNA or translation of target protein. As an example, the efficiency of inhibition may be determined by assessing the amount of gene product in the cell: mRNA may be detected with a hybridization probe having a nucleotide sequence outside the region used for the inhibitory double-stranded RNA, or translated polypeptide may be detected with an antibody raised against the polypeptide sequence of that region.

The RNA may comprise one or more strands of polymerized ribonucleotide. It may include modifications to either the phosphate-sugar backbone or the nucleoside. For example, the phosphodiester linkages of natural RNA may be modified to include at least one of a nitrogen or sulfur heteroatom. Modifications in RNA structure may be tailored

to allow specific genetic inhibition while avoiding a general panic response in some organisms which is generated by dsRNA. Likewise, bases may be modified to block the activity of adenosine deaminase. RNA may be produced enzymatically or by partial/total organic synthesis, any modified ribonucleotide can be introduced by *in vitro* enzymatic or organic synthesis.

The double-stranded structure may be formed by a single self-complementary RNA strand or two complementary RNA strands. RNA duplex formation may be initiated either inside or outside the cell. The RNA may be introduced in an amount which allows delivery of at least one copy per cell. Higher doses (e.g., at least 5, 10, 100, 500 or 1000 copies per cell) of double-stranded material may yield more effective inhibition; lower doses may also be useful for specific applications. Inhibition is sequence-specific in that nucleotide sequences corresponding to the duplex region of the RNA are targeted for genetic inhibition.

RNA containing a nucleotide sequences identical to a portion of the target gene are preferred for inhibition. RNA sequences with insertions, deletions, and single point mutations relative to the target sequence have also been found to be effective for inhibition. Thus, sequence identity may optimized by sequence comparison and alignment algorithms known in the art (see Gribskov and Devereux, *Sequence Analysis Primer*, Stockton Press, 1991, and references cited therein) and calculating the percent difference between the nucleotide sequences by, for example, the Smith-Waterman algorithm as implemented in the BESTFIT software program using default parameters (e.g., University of Wisconsin Genetic Computing Group). Greater than 90% sequence identity, or even 100% sequence identity, between the inhibitory RNA and the portion of the target gene is preferred. Alternatively, the duplex region of the RNA may be defined functionally as a nucleotide sequence that is capable of hybridizing with a portion of the target gene transcript (e.g., 400 mM NaCl, 40 mM PIPES pH 6.4, 1 mM EDTA, 50°C or 70°C hybridization for 12-16 hours; followed by washing). The length of the identical nucleotide sequences may be at least 25. 50, 100, 200, 300 or 400 bases.

As disclosed herein, 100% sequence identity between the RNA and the target gene is not required to practice the present invention. Thus the invention has the advantage of

being able to tolerate sequence variations that might be expected due to genetic mutation, strain polymorphism, or evolutionary divergence.

The cell with the target gene may be derived from or contained in any organism. The organism may a plant, animal, protozoan, bacterium, virus, or fungus. The plant may be a monocot, dicot or gymnosperm; the animal may be a vertebrate or invertebrate. Preferred microbes are those used in agriculture or by industry, and those that are pathogenic for plants or animals. Fungi include organisms in both the mold and yeast morphologies.

Plants include arabidopsis; field crops (e.g., alfalfa, barley, bean, corn, cotton, flax, pea, rape, rice, rye, safflower, sorghum, soybean, sunflower, tobacco, and wheat); vegetable crops (e.g., asparagus, beet, broccoli, cabbage, carrot, cauliflower, celery, cucumber, eggplant, lettuce, onion, pepper, potato, pumpkin, radish, spinach, squash, taro, tomato, and zucchini); fruit and nut crops (e.g., almond, apple, apricot, banana, blackberry, blueberry, cacao, cherry, coconut, cranberry, date, fajoa, filbert, grape, grapefruit, guava, kiwi, lemon, lime, mango, melon, nectarine, orange, papaya, passion fruit, peach, peanut, pear, pineapple, pistachio, plum, raspberry, strawberry, tangerine, walnut, and watermelon); and ornamentals (e.g., alder, ash, aspen, azalea, birch, boxwood, camellia, carnation, chrysanthemum, elm, fir, ivy, jasmine, juniper, oak, palm, poplar, pine, redwood, rhododendron, rose, and rubber).

Examples of vertebrate animals include fish, mammal, cattle, goat, pig, sheep, rodent, hamster, mouse, rat, primate, and human; invertebrate animals include nematodes, other worms, drosophila, and other insects. Representative generae of nematodes include those that infect animals (e.g., Ancylostoma, Ascaridia, Ascaris, Bunostomum, Caenorhabditis, Capillaria, Chabertia, Cooperia, Dictyocaulus, Haemonchus, Heterakis, Nematodirus, Oesophagostomum, Ostertagia, Oxyuris, Parascaris, Strongylus, Toxascaris, Trichuris, Trichostrongylus, Tfhchonema, Toxocara, Uncinaria) and those that infect plants (e.g., Bursaphalenchus, Criconemella, Diiylenchus, Ditylenchus, Globodera, Helicotylenchus, Heterodera, Longidorus, Melodoigyne, Nacobbus, Paratylenchus, Pratylenchus, Radopholus, Rotelynchus, Tylenchus, and Xiphinema). Representative orders of insects include Coleoptera, Diptera, Lepidoptera, and Homoptera.

The cell having the target gene may be from the germ line or somatic, totipotent or pluripotent, dividing or non-dividing, parenchyma or epithelium, immortalized or transformed, or the like. The cell may be a stem cell or a differentiated cell. Cell types that are differentiated include adipocytes, fibroblasts, myocytes, cardiomyocytes, endothelium, neurons, glia, blood cells, megakaryocytes, lymphocytes, macrophages, neutrophils, eosinophils, basophils, mast cells, leukocytes, granulocytes, keratinocytes, chondrocytes, osteoblasts, osteoclasts, hepatocytes, and cells of the endocrine or exocrine glands.

RNA may be synthesized either *in vivo* or *in vitro*. Endogenous RNA polymerase of the cell may mediate transcription *in vivo*, or cloned RNA polymerase can be used for transcription *in vivo* or in *vitro*. For transcription from a transgene *in vivo* or an expression construct, a regulatory region (e.g., promoter, enhancer, silencer, splice donor and acceptor, polyadenylation) may be used to transcribe the RNA strand (or strands). Inhibition may be targeted by specific transcription in an organ, tissue, or cell type; stimulation of an environmental condition (e.g., infection, stress, temperature, chemical inducers); and/or engineering transcription at a developmental stage or age. The RNA strands may or may not be polyadenylated; the RNA strands may or may not be capable of being translated into a polypeptide by a cell's translational apparatus. RNA may be chemically or enzymatically synthesized by manual or automated reactions. The RNA may be synthesized by a cellular RNA polymerase or a bacteriophage RNA polymerase (e.g., T3, T7, SP6). The use and production of an expression construct are known in the art[32, 33, 34] (see also WO 97/32016; U.S. Pat. Nos. 5,593,874, 5,698,425, 5,712,135, 5,789,214, and 5,804,693; and the references cited therein). If synthesized chemically or by *in vitro* enzymatic synthesis, the RNA may be purified prior to introduction into the cell. For example, RNA can be purified from a mixture by extraction with a solvent or resin, precipitation, electrophoresis, chromatography, or a combination thereof. Alternatively, the RNA may be used with no or a minimum of purification to avoid losses due to sample processing. The RNA may be dried for storage or dissolved in an aqueous solution. The solution may contain buffers or salts to promote annealing, and/or stabilization of the duplex strands.

RNA may be directly introduced into the cell (i.e., intracellularly); or introduced extracellularly into a cavity, interstitial space, into the circulation of an organism, intro-

duced orally, or may be introduced by bathing an organism in a solution containing the RNA. Methods for oral introduction include direct mixing of the RNA with food of the organism, as well as engineered approaches in which a species that is used as food is engineered to express the RNA, then fed to the organism to be affected. For example, the RNA may be sprayed onto a plant or a plant may be genetically engineered to express the RNA in an amount sufficient to kill some or all of a pathogen known to infect the plant. Physical methods of introducing nucleic acids, for example, injection directly into the cell or extracellular injection into the organism, may also be used. We disclose herein that in *C. elegans*, double-stranded RNA introduced outside the cell inhibits gene expression. Vascular or extravascular circulation, the blood or lymph system, the phloem, the roots, and the cerebrospinal fluid are sites where the RNA may be introduced. A transgenic organism that expresses RNA from a recombinant construct may be produced by introducing the construct into a zygote, an embryonic stem cell, or another multipotent cell derived from the appropriate organism.

Physical methods of introducing nucleic acids include injection of a solution containing the RNA, bombardment by particles covered by the RNA, soaking the cell or organism in a solution of the RNA, or electroporation of cell membranes in the presence of the RNA. A viral construct packaged into a viral particle would accomplish both efficient introduction of an expression construct into the cell and transcription of RNA encoded by the expression construct. Other methods known in the art for introducing nucleic acids to cells may be used, such as lipid-mediated carrier transport, chemical-mediated transport, such as calcium phosphate, and the like. Thus the RNA may be introduced along with components that perform one or more of the following activities: enhance RNA uptake by the cell, promote annealing of the duplex strands, stabilize the annealed strands, or other-wise increase inhibition of the target gene.

The present invention may be used to introduce RNA into a cell for the treatment or prevention of disease. For example, dsRNA may be introduced into a cancerous cell or tumor and thereby inhibit gene expression of a gene required for maintenance of the carcinogenic/tumorigenic phenotype. To prevent a disease or other pathology, a target gene may be selected which is required for initiation or maintenance of the disease/pathology.

Treatment would include amelioration of any symptom associated with the disease or clinical indication associated with the pathology.

A gene derived from any pathogen may be targeted for inhibition. For example, the gene could cause immunosuppression of the host directly or be essential for replication of the pathogen, transmission of the pathogen, or maintenance of the infection. The inhibitory RNA could be introduced in cells *in vitro* or *ex vivo* and then subsequently placed into an animal to affect therapy, or directly treated by *in vivo* administration. A method of gene therapy can be envisioned. For example, cells at risk for infection by a pathogen or already infected cells, particularly human immunodeficiency virus (HIV) infections, may be targeted for treatment by introduction of RNA according to the invention. The target gene might be a pathogen or host gene responsible for entry of a pathogen into its host, drug metabolism by the pathogen or host, replication or integration of the pathogen's genome, establishment or spread of an infection in the host, or assembly of the next generation of pathogen. Methods of prophylaxis (i.e., prevention or decreased risk of infection), as well as reduction in the frequency or severity of symptoms associated with infection, can be envisioned.

The present invention could be used for treatment or development of treatments for cancers of any type, including solid tumors and leukemias, including: apudoma, choristoma, branchioma, malignant carcinoid syndrome, carcinoid heart disease, carcinoma (e.g., Walker, basal cell, basosquamous, Brown-Pearce, ductal, Ehrlich tumor, in situ, Krebs 2, Merkel cell, mucinous, non-small cell lung, oat cell, papillary, scirrhous, bronchiolar, bronchogenic, squamous cell, and transitional cell), histiocytic disorders, leukemia (e.g., B cell, mixed cell, null cell, T cell, T-cell chronic, HTLV-II-associated, lymphocytic acute, lymphocytic chronic, mast cell, and myeloid), histiocytosis malignant, Hodgkin disease, immunoproliferative small, non-Hodgkin lymphoma, plasmacytoma, reticuloendotheliosis, melanoma, chondroblastoma, chondroma, chondrosarcoma, fibroma, fibrosarcoma, giant cell tumors, histiocytoma, lipoma, liposarcoma, mesothelioma, myxoma, myxosarcoma, osteoma, osteosarcoma, Ewing sarcoma, synovioma, adenofibroma, adenolymphoma, carcinosarcoma, chordoma, cranio-pharyngioma, dysgerminoma, hamartoma, mesenchymoma, mesonephroma, myosarcoma, ameloblastoma, cementoma, odontoma, teratoma, thymoma, trophoblastic tumor, adeno-

carcinoma, adenoma, cholangioma, cholesteatoma, cylindroma, cystadenocarcinoma, cystadenoma, granulosa cell tumor, gynandroblastoma, hepatoma, hidradenoma, islet cell tumor, Leydig cell tumor, papilloma, Sertoli cell tumor, theca cell tumor, leiomyoma, leiomyosarcoma, myoblastoma, myoma, myosarcoma, rhabdomyoma, rhabdomyosarcoma, ependymoma, ganglioneuroma, glioma, medulloblastoma, meningioma, neurilemmoma, neuroblastoma, neuroepithelioma, neurofibroma, neuroma, paraganglioma, paraganglioma nonchromaffin, angiokeratoma, angiolymphoid hyperplasia with eosinophilia, angioma sclerosing, angiomatosis, glomangioma, hemangioendothelioma, hemangioma, hemangiopericytoma, hemangiosarcoma, lymphangioma, lymphangiomyoma, lymphangiosarcoma, pinealoma, carcinosarcoma, chondrosarcoma, cystosarcoma phyllodes, fibrosarcoma, hemangiosarcoma, leiomyosarcoma, leukosarcoma, liposarcoma, lymphangiosarcoma, myosarcoma, myxosarcoma, ovarian carcinoma, rhabdomyosarcoma, sarcoma (e.g., Ewing, experimental, Kaposi, and mast cell), neoplasms (e.g., bone, breast, digestive system, colorectal, liver, pancreatic, pituitary, testicular, orbital, head and neck, central nervous system, acoustic, pelvic, respiratory tract, and urogenital), neurofibromatosis, and cervical dysplasia, and for treatment of other conditions in which cells have become immortalized or transformed. The invention could be used in combination with other treatment modalities, such as chemotherapy, cryotherapy, hyperthermia, radiation therapy, and the like.

As disclosed herein, the present invention may is not limited to any type of target gene or nucleotide sequence. But the following classes of possible target genes are listed for illustrative purposes: developmental genes (e.g., adhesion molecules, cyclin kinase inhibitors, Wnt family members, Pax family members, Winged helix family members, Hox family members, cytokines/lymphokines and their receptors, growth/differentiation factors and their receptors, neurotransmitters and their receptors); oncogenes (e.g., ABL1, BCL1, BCL2, BCL6, CBFA2, CBL, CSF1R, ERBA, ERBB, EBRB2, ETS1, ETS1, ETV6, FGR, FOS, FYN, HCR, HRAS, JUN, KRAS, LCK, LYN, MDM2, MLL, MYB, MYC, MYCL1, MYCN, NRAS, PIM1, PML, RET, SRC, TAL1, TCL3, and YES); tumor suppressor genes (e.g., APC, BRCA1, BRCA2, MADH4, MCC, NF1, NF2, RB1, TP53, and WT1); and enzymes (e.g., ACC synthases and oxidases, ACP desaturases and hydroxylases, ADP-glucose pyrophorylases, ATPases, alcohol dehydrogenases, amylases,

amyloglucosidases, catalases, cellulases, chalcone synthases, chitinases, cyclooxygenases, decarboxylases, dextrinases, DNA and RNA polymerases, galactosidases, glucanases, glucose oxidases, granule-bound starch synthases, GTPases, helicases, hemicellulases, integrases, inulinases, invertases, isomerases, kinases, lactases, lipases, lipoxygenases, lysozymes, nopaline synthases, octopine synthases, pectinesterases, peroxidases, phosphatases, phospholipases, phosphorylases, phytases, plant growth regulator synthases, polygalacturonases, proteinases and peptidases, pullanases, recombinases, reverse transcriptases, RUBISCOs, topoisomerases, and xylanases).

The present invention could comprise a method for producing plants with reduced susceptibility to climatic injury, susceptibility to insect damage, susceptibility to infection by a pathogen, or altered fruit ripening characteristics. The targeted gene may be an enzyme, a plant structural protein, a gene involved in pathogenesis, or an enzyme that is involved in the production of a non-proteinaceous part of the plant (i.e., a carbohydrate or lipid). If an expression construct is used to transcribe the RNA in a plant, transcription by a wound- or stress-inducible; tissue-specific (e.g., fruit, seed, anther, flower, leaf, root); or otherwise regulatable (e.g., infection, light, temperature, chemical) promoter may be used. By inhibiting enzymes at one or more points in a metabolic pathway or genes involved in pathogenesis, the effect may be enhanced: each activity will be affected and the effects may be magnified by targeting multiple different components. Metabolism may also be manipulated by inhibiting feedback control in the pathway or production of unwanted metabolic byproducts.

The present invention may be used to reduce crop destruction by other plant pathogens such as arachnids, insects, nematodes, protozoans, bacteria, or fungi. Some such plants and their pathogens are listed in *Index of Plant Diseases in the United States* (U.S. Dept. of Agriculture Handbook No. 165, 1960); *Distribution of Plant-Parasitic Nematode Species in North America* (Society of Nematologists, 1985); and *Fungi on Plants and Plant Products in the United States* (American Phytopathological Society, 1989). Insects with reduced ability to damage crops or improved ability to prevent other destructive insects from damaging crops may be produced. Furthermore, some nematodes are vectors of plant pathogens, and may be attacked by other beneficial nematodes which have no effect on plants. Inhibition of target gene activity could be used to delay or

prevent entry into a particular developmental step (e.g., metamorphosis), if plant disease was associated with a particular stage of the pathogen's life cycle. Interactions between pathogens may also be modified by the invention to limit crop damage. For example, the ability of beneficial nematodes to attack their harmful prey may be enhanced by inhibition of behavior-controlling nematode genes according to the invention.

Although pathogens cause disease, some of the microbes interact with their plant host in a beneficial manner. For example, some bacteria are involved in symbiotic relationships that fix nitrogen and some fungi produce phytohormones. Such beneficial interactions may be promoted by using the present invention to inhibit target gene activity in the plant and/or the microbe.

Another utility of the present invention could be a method of identifying gene function in an organism comprising the use of double-stranded RNA to inhibit the activity of a target gene of previously unknown function. Instead of the time consuming and laborious isolation of mutants by traditional genetic screening, functional genomics would envision determining the function of uncharacterized genes by employing the invention to reduce the amount and/or alter the timing of target gene activity. The invention could be used in determining potential targets for pharmaceutics, understanding normal and pathological events associated with development, determining signaling pathways responsible for postnatal development/aging, and the like. The increasing speed of acquiring nucleotide sequence information from genomic and expressed gene sources, including total sequences for the yeast, *D. melanogaster*, and *C. elegans* genomes, can be coupled with the invention to determine gene function in an organism (e.g., nematode). The preference of different organisms to use particular codons, searching sequence databases for related gene products, correlating the linkage map of genetic traits with the physical map from which the nucleotide sequences are derived, and artificial intelligence methods may be used to define putative open reading frames from the nucleotide sequences acquired in such sequencing projects.

A simple assay would be to inhibit gene expression according to the partial sequence available from an expressed sequence tag (EST). Functional alterations in growth, development, metabolism, disease resistance, or other biological processes would be indicative of the normal role of the EST's gene product.

The ease with which RNA can be introduced into an intact cell/organism containing the target gene allows the present invention to be used in high throughput screening (HTS). For example, duplex RNA can be produced by an amplification reaction using primers flanking the inserts of any gene library derived from the target cell/organism. Inserts may be derived from genomic DNA or mRNA (e.g., cDNA and cRNA). Individual clones from the library can be replicated and then isolated in separate reactions, but preferably the library is maintained in individual reaction vessels (e.g., a 96-well microtiter plate) to minimize the number of steps required to practice the invention and to allow automation of the process. Solutions containing duplex RNAs that are capable of inhibiting the different expressed genes can be placed into individual wells positioned on a microtiter plate as an ordered array, and intact cells/organisms in each well can be assayed for any changes or modifications in behavior or development due to inhibition of target gene activity. The amplified RNA can be fed directly to, injected into, the cell/organism containing the target gene. Alternatively, the duplex RNA can be produced by *in vivo* or *in vitro* transcription from an expression construct used to produce the library. The construct can be replicated as individual clones of the library and transcribed to produce the RNA; each clone can then be fed to, or injected into, the cell/organism containing the target gene. The function of the target gene can be assayed from the effects it has on the cell/organism when gene activity is inhibited. This screening could be amenable to small subjects that can be processed in large number, for example: arabidopsis, bacteria, drosophila, fungi, nematodes, viruses, zebrafish, and tissue culture cells derived from mammals.

A nematode or other organism that produces a colorimetric, fluorogenic, or luminescent signal in response to a regulated promoter (e.g., transfected with a reporter gene construct) can be assayed in an HTS format to identify DNA-binding proteins that regulate the promoter. In the assay's simplest form, inhibition of a negative regulator results in an increase of the signal and inhibition of a positive regulator results in a decrease of the signal.

If a characteristic of an organism is determined to be genetically linked to a polymorphism through RFLP or QTL analysis, the present invention can be used to gain insight regarding whether that genetic polymorphism might be directly responsible for the

characteristic. For example, a fragment defining the genetic polymorphism or sequences in the vicinity of such a genetic polymorphism can be amplified to produce an RNA, the duplex RNA can be introduced to the organism, and whether an alteration in the characteristic is correlated with inhibition can be determined. Of course, there may be trivial explanations for negative results with this type of assay, for example: inhibition of the target gene causes lethality, inhibition of the target gene may not result in any observable alteration, the fragment contains nucleotide sequences that are not capable of inhibiting the target gene, or the target gene's activity is redundant.

The present invention may be useful in allowing the inhibition of essential genes. Such genes may be required for cell or organism viability at only particular stages of development or cellular compartments. The functional equivalent of conditional mutations may be produced by inhibiting activity of the target gene when or where it is not required for viability. The invention allows addition of RNA at specific times of development and locations in the organism without introducing permanent mutations into the target genome.

If alternative splicing produced a family of transcripts that were distinguished by usage of characteristic exons, the present invention can target inhibition through the appropriate exons to specifically inhibit or to distinguish among the functions of family members. For example, a hormone that contained an alternatively spliced transmembrane domain may be expressed in both membrane bound and secreted forms. Instead of isolating a nonsense mutation that terminates translation before the transmembrane domain, the functional consequences of having only secreted hormone can be determined according to the invention by targeting the exon containing the transmembrane domain and thereby inhibiting expression of membrane-bound hormone.

The present invention may be used alone or as a component of a kit having at least one of the reagents necessary to carry out the *in vitro* or *in vivo* introduction of RNA to test samples or subjects. Preferred components are the dsRNA and a vehicle that promotes introduction of the dsRNA. Such a kit may also include instructions to allow a user of the kit to practice the invention.

Pesticides may include the RNA molecule itself, an expression construct capable of expressing the RNA, or organisms transfected with the expression construct. The

pesticide of the present invention may serve as an arachnicide, insecticide, nematicide, viricide, bactericide, and/or fungicide. For example, plant parts that are accessible above ground (e.g., flowers, fruits, buds, leaves, seeds, shoots, bark, stems) may be sprayed with pesticide, the soil may be soaked with pesticide to access plant parts growing beneath ground level, or the pest may be contacted with pesticide directly. If pests interact with each other, the RNA may be transmitted between them. Alternatively, if inhibition of the target gene results in a beneficial effect on plant growth or development, the aforementioned RNA, expression construct, or transfected organism may be considered a nutritional agent. In either case, genetic engineering of the plant is not required to achieve the objectives of the invention.

Alternatively, an organism may be engineered to produce dsRNA which produces commercially or medically beneficial results, for example, resistance to a pathogen or its pathogenic effects, improved growth, or novel developmental patterns.

Used as either an pesticide or nutrient, a formulation of the present invention may be delivered to the end user in dry or liquid form: for example, as a dust, granulate, emulsion, paste, solution, concentrate, suspension, or encapsulation. Instructions for safe and effective use may also be provided with the formulation. The formulation might be used directly, but concentrates would require dilution by mixing with an extender provided by the formulator or the end user. Similarly, an emulsion, paste, or suspension may require the end user to perform certain preparation steps before application. The formulation may include a combination of chemical additives known in the art such as solid carriers, minerals, solvents, dispersants, surfactants, emulsifiers, tackifiers, binders, and other adjuvants. Preservatives and stabilizers may also be added to the formulation to facilitate storage. The crop area or plant may also be treated simultaneously or separately with other pesticides or fertilizers. Methods of application include dusting, scattering or pouring, soaking, spraying, atomizing, and coating. The precise physical form and chemical composition of the formulation, and its method of application, would be chosen to promote the objectives of the invention and in accordance with prevailing circumstances. Expression constructs and transfected hosts capable of replication may also promote the persistence and/or spread of the formulation.

Description of the dsRNA Inhibition Phenomenon in *C. elegans*

The operation of the present invention was shown in the model genetic organism *Caenorhabditis elegans*.

Introduction of RNA into cells had been seen in certain biological systems to interfere with function of an endogenous gene[1,2]. Many such effects were believed to result from a simple antisense mechanism dependent on hybridization between injected single-stranded RNA and endogenous transcripts. In other cases, a more complex mechanism had been suggested. One instance of an RNA-mediated mechanism was RNA interference (RNAi) phenomenon in the nematode *C. elegans*. RNAi had been used in a variety of studies to manipulate gene expression[3,4].

Despite the usefulness of RNAi in *C. elegans*, many features had been difficult to explain. Also, the lack of a clear understanding of the critical requirements for interfering RNA led to a sporadic record of failure and partial success in attempts to extend RNAi beyond the earliest stages following injection. A statement frequently made in the literature was that sense and antisense RNA preparations are each sufficient to cause interference[3,4]. The only precedent for such a situation was in plants where the process of co-suppression had a similar history of usefulness in certain cases, failure in others, and no ability to design interference protocols with a high chance of success. Working with *C. elegans*, we discovered an RNA structure that would give effective and uniform genetic inhibition. The prior art did not teach or suggest that RNA structure was a critical feature for inhibition of gene expression. Indeed the ability of crude sense and antisense preparations to produce interference[3,4] had been taken as an indication that RNA structure was not a critical factor. Instead, the extensive plant literature and much of the ongoing research in *C. elegans* was focused on the possibility that detailed features of the target gene sequence or its chromosomal locale was the critical feature for interfering with gene expression.

The inventors carefully purified sense or antisense RNA for *unc-22* and tested each for gene-specific inhibition. While the crude sense and antisense preparations had strong interfering activity, it was found that the purified sense and antisense RNAs had only marginal inhibitory activity. This was unexpected because many techniques in molecular biology are based on the assumption that RNA produced with specific *in vitro*

promoters (e.g., T3 or T7 RNA polymerase), or with characterized promoters *in vivo*, is produced predominantly from a single strand. The inventors had carried out purification of these crude preparations to investigate whether a small fraction of the RNA had an unusual structure which might be responsible for the observed genetic inhibition. To rigorously test whether double-stranded character might contribute to genetic inhibition, the inventors carried out additional purification of single-stranded RNAs and compared inhibitory activities of individual strands with that of the double-stranded hybrid.

The following examples are meant to be illustrative of the present invention; however, the practice of the invention is not limited or restricted in any way by them.

Analysis of RNA-Mediated Inhibition of *C. elegans* Genes

The *unc-22* gene was chosen for initial comparisons of activity as a result of previous genetic analysis that yields a semi-quantitative comparison between *unc-22* gene activity and the movement phenotypes of animals[3,8]: decreases in activity produce an increasingly severe twitching phenotype, while complete loss of function results in the additional appearance of muscle structural defects and impaired motility. *unc-22* encodes an abundant but non-essential myofilament protein[7-9]. *unc-22* mRNA is present at several thousand copies per striated muscle cell[3].

Purified antisense and sense RNAs covering a 742 nt segment of *unc-22* had only marginal inhibitory activity, requiring a very high dose of injected RNA for any observable effect (Figure 4). By contrast, a sense+antisense mixture produced a highly effective inhibition of endogenous gene activity (Figure 4). The mixture was at least two orders of magnitude more effective than either single strand in inhibiting gene expression. The lowest dose of the sense+antisense mixture tested, approximately 60,000 molecules of each strand per adult, led to twitching phenotypes in an average of 100 progeny. *unc-22* expression begins in embryos with approximately 500 cells. At this point, the original injected material would be diluted to at most a few molecules per cell.

The potent inhibitory activity of the sense+antisense mixture could reflect formation of double-stranded RNA (dsRNA), or conceivably some alternate synergy between the strands. Electrophoretic analysis indicated that the injected material was predomi-

nantly double stranded. The dsRNA was gel purified from the annealed mixture and found to retain potent inhibitory activity. Although annealing prior to injection was compatible with inhibition, it was not necessary. Mixing of sense and antisense RNAs in low salt (under conditions of minimal dsRNA formation), or rapid sequential injection of sense and antisense strands, were sufficient to allow complete inhibition. A long interval (>1 hour) between sequential injections of sense and antisense RNA resulted in a dramatic decrease in inhibitory activity. This suggests that injected single strands may be degraded or otherwise rendered inaccessible in the absence of the complementary strand.

An issue of specificity arises when considering known cellular responses to dsRNA. Some organisms have a dsRNA-dependent protein kinase that activates a panic response mechanism[10]. Conceivably, the inventive sense+antisense synergy could reflect a non-specific potentiation of antisense effects by such a panic mechanism. This was not found to be the case: co-injection of dsRNA segments unrelated to *unc-22* did not potentiate the ability of *unc-22* single strands to mediate inhibition. Also investigated was whether double-stranded structure could potentiate inhibitory activity when placed in *cis* to a single-stranded segment. No such potentiation was seen; unrelated double-stranded sequences located 5' or 3' of a single-stranded *unc-22* segment did not stimulate inhibition. Thus potentiation of gene-specific inhibition was observed only when dsRNA sequences exist within the region of homology with the target gene.

The phenotype produced by *unc-22* dsRNA was specific. Progeny of injected animals exhibited behavior indistinguishable from characteristic *unc-22* loss of function mutants. Target-specificity of dsRNA effects using three additional genes with well characterized phenotypes (Figure 1 and Table 1). *unc-54* encodes a body wall muscle myosin heavy chain isoform required for full muscle contraction[7,11,12], *fem-1* encodes an ankyrin-repeat containing protein required in hermaphrodites for sperm production[13,14], and *hlh-1* encodes a *C. elegans* homolog of the myoD family required for proper body shape and motility[15,16]. For each of these genes, injection of dsRNA produced progeny broods exhibiting the known null mutant phenotype, while the purified single strands produced no significant reduction in gene expression. With one exception, all of the phenotypic consequences of dsRNA injection were those expected from inhibition of the corresponding gene. The exception (segment *unc54C*, which led to an embryonic and

larval arrest phenotype not seen with *unc-54* null mutants) was illustrative. This segment covers the highly conserved myosin motor domain, and might have been expected to inhibit the activity of other highly related myosin heavy chain genes[17]. This interpretation would support uses of the present invention in which nucleotide sequence comparison of dsRNA and target gene show less than 100% identity. The *unc54C* segment has been unique in our overall experience to date: effects of 18 other dsRNA segments have all been limited to those expected from characterized null mutants.

The strong phenotypes seen following dsRNA injection are indicative of inhibitory effects occurring in a high fraction of cells. The *unc-54* and *hlh-1* muscle phenotypes, in particular, are known to result from a large number of defective muscle cells[11,16]. To examine inhibitory effects of dsRNA on a cellular level, a transgenic line expressing two different GFP-derived fluorescent reporter proteins in body muscle was used. Injection of dsRNA directed to *gfp* produced dramatic decreases in the fraction of fluorescent cells (Figure 2). Both reporter proteins were absent from the negative cells, while the few positive cells generally expressed both GFP forms.

The pattern of mosaicism observed with *gfp* inhibition was not random. At low doses of dsRNA, the inventors saw frequent inhibition in the embryonically-derived muscle cells present when the animal hatched. The inhibitory effect in these differentiated cells persisted through larval growth: these cells produced little or no additional GFP as the affected animals grew. The 14 postembryonically-derived striated muscles are born during early larval stages and were more resistant to inhibition. These cells have come through additional divisions (13-14 versus 8-9 for embryonic muscles[18,19]). At high concentrations of *gfp* dsRNA, inhibition was noted in virtually all striated bodywall muscles, with occasional single escaping cells including cells born in embryonic or post-embryonic stages. The nonstriated vulval muscles, born during late larval development, appeared resistant to genetic inhibition at all tested concentrations of injected RNA. The latter result is important for evaluating the use of the present invention in other systems. First, it indicates that failure in one set of cells from an organism does not necessarily indicate complete non-applicability of the invention to that organism. Second, it is important to realize that not all tissues in the organism need to be affected for the invention to be used in an organism. This may serve as an advantage in some situations.

A few observations serve to clarify the nature of possible targets and mechanisms for RNA-mediated genetic inhibition in *C. elegans*:

First, dsRNA segments corresponding to a variety of intron and promoter sequences did not produce detectable inhibition (Table 1). Although consistent with possible inhibition at a post-transcriptional level, these experiments do not rule out inhibition at the level of the gene.

Second, dsRNA injection produced a dramatic decrease in the level of the endogenous mRNA transcript (Figure 3). Here, a *mex-3* transcript that is abundant in the gonad and early embryos[20] was targeted, where straightforward *in situ* hybridization can be performed[5]. No endogenous *mex-3* mRNA was observed in animals injected with a dsRNA segment derived from *mex-3* (Figure 3D), but injection of purified *mex-3* antisense RNA resulted in animals that retained substantial endogenous mRNA levels (Figure 3C).

Third, dsRNA-mediated inhibition showed a surprising ability to cross cellular boundaries. Injection of dsRNA for *unc-22*, *gfp*, or *lacZ* into the body cavity of the head or tail produced a specific and robust inhibition of gene expression in the progeny brood (Table 2). Inhibition was seen in the progeny of both gonad arms, ruling out a transient "nicking" of the gonad in these injections. dsRNA injected into body cavity or gonad of young adults also produced gene-specific inhibition in somatic tissues of the injected animal (Table 2).

Table 3 shows that *C. elegans* can respond in a gene-specific manner to dsRNA encountered in the environment. Bacteria are a natural food source for *C. elegans*. The bacteria are ingested, ground in the animal's pharynx, and the bacterial contents taken up in the gut. The results show that *E. coli* bacteria expressing dsRNAs can confer specific inhibitory effects on *C. elegans* nematode larvae that feed on them.

Three *C. elegans* genes were analyzed. For each gene, corresponding dsRNA was expressed in *E. coli* by inserting a segment of the coding region into a plasmid construct designed for bidirectional transcription by bacteriophage T7 RNA polymerase. The dsRNA segments used for these experiments were the same as those used in previous microinjection experiments (see Figure 1). The effects resulting from feeding these bacteria to *C. elegans* were compared to the effects achieved by microinjecting animals

with dsRNA.

The *C. elegans* gene *unc-22* encodes an abundant muscle filament protein. *unc-22* null mutations produce a characteristic and uniform twitching phenotype in which the animals can sustain only transient muscle contraction. When wild-type animals were fed bacteria expressing a dsRNA segment from *unc-22*, a high fraction (85%) exhibited a weak but still distinct twitching phenotype characteristic of partial loss of function for the *unc-22* gene. The *C. elegans fem-1* gene encodes a late component of the sex determination pathway. Null mutations prevent the production of sperm and lead euploid (XX) animals to develop as females, while wild type XX animals develop as hermaphrodites. When wild-type animals were fed bacteria expressing dsRNA corresponding to *fem-1*, a fraction (43%) exhibit a sperm-less (female) phenotype and were sterile. Finally, the ability to inhibit gene expression of a transgene target was assessed. When animals carrying a *gfp* transgene were fed bacteria expressing dsRNA corresponding to the *gfp* reporter, an obvious decrease in the overall level of GFP fluorescence was observed, again in approximately 12% of the population (see Figure 5, panels B and C).

The effects of these ingested RNAs were specific. Bacteria carrying different dsRNAs from *fem-1* and *gfp* produced no twitching, dsRNAs from *unc-22* and *fem-1* did not reduce *gfp* expression, and dsRNAs from *gfp* and *unc-22* did not produce females. These inhibitory effects were apparently mediated by dsRNA: bacteria expressing only the sense or antisense strand for either *gfp* or *unc-22* caused no evident phenotypic effects on their *C. elegans* predators.

Table 4 shows the effects of bathing *C. elegans* in a solution containing dsRNA. Larvae were bathed for 24 hours in solutions of the indicated dsRNAs (1 mg/ml), then allowed to recover in normal media and allowed to grow under standard conditions for two days. The *unc-22* dsRNA was segment ds-*unc22A* from Figure 1. *pos*-1 and *sqt*-3 dsRNAs were from the full length cDNA clones. *pos-1* encodes an essential maternally provided component required early in embyogenesis. Mutations removing *pos-1* activity have an early embryonic arrest characteristic of *skn*-like mutations[29, 30]. Cloning and activity patterns for *sqt-3* have been described[31]. *C. elegans sqt-3* mutants have mutations in the *col-1* collagen gene[31]. Phenotypes of affected animals are noted. Incidences of

clear phenotypic effects in these experiments were 5-10% for *unc-22*, 50% for *pos-1*, and 5% for *sqt-3*. These are frequencies of unambiguous phenocopies; other treated animals may have had marginal defects corresponding to the target gene that were not observable. Each treatment was fully gene-specific in that *unc-22* dsRNA produced only Unc-22 phenotypes, *pos-1* dsRNA produced only Pos-1 phenotypes, and *sqt-3* dsRNA produced only Sqt-3 phenotypes.

Some of the results described herein were published after the filing of our provisional application. Those publications and a review can be cited as Fire, A., et al. Nature, 391, 806-811, 1998; Timmons, L. & Fire, A. Nature, 395, 854, 1998; and Montgomery, M.K. & Fire, A. Trends in Genetics, 14, 255-258, 1998.

The effects described herein significantly augment available tools for studying gene function in *C. elegans* and other organisms. In particular, functional analysis should now be possible for a large number of interesting coding regions[21] for which no specific function have been defined. Several of these observations show the properties of dsRNA that may affect the design of processes for inhibition of gene expression. For example, one case was observed in which a nucleotide sequence shared between several myosin genes may inhibit gene expression of several members of a related gene family.

Methods of RNA Synthesis and Microinjection

RNA was synthesized from phagemid clones with T3 and T7 RNA polymerase[6], followed by template removal with two sequential DNase treatments. In cases where sense, antisense, and mixed RNA populations were to be compared, RNAs were further purified by electrophoresis on low-gelling-temperature agarose. Gel-purified products appeared to lack many of the minor bands seen in the original "sense" and "antisense" preparations. Nonetheless, RNA species accounting for less than 10% of purified RNA preparations would not have been observed. Without gel purification, the "sense" and "antisense" preparations produced significant inhibition. This inhibitory activity was reduced or eliminated upon gel purification. By contrast, sense+antisense mixtures of gel purified and non-gel-purified RNA preparations produced identical effects.

Following a short (5 minute) treatment at 68°C to remove secondary structure, sense+antisense annealing was carried out in injection buffer[27] at 37°C for 10-30 minutes.

Formation of predominantly double stranded material was confirmed by testing migration on a standard (non-denaturing) agarose gel: for each RNA pair, gel mobility was shifted to that expected for double-stranded RNA of the appropriate length. Co-incubation of the two strands in a low-salt buffer (5 mM Tris-HCl pH 7.5, 0.5 mM EDTA) was insufficient for visible formation of double-stranded RNA *in vitro*. Non-annealed sense+antisense RNAs for *unc22B* and *gfpG* were tested for inhibitory effect and found to be much more active than the individual single strands, but 2-4 fold less active than equivalent pre-annealed preparations.

After pre-annealing of the single strands for *unc22A*, the single electrophoretic species corresponding in size to that expected for dsRNA was purified using two rounds of gel electrophoresis. This material retained a high degree of inhibitory activity.

Except where noted, injection mixes were constructed so animals would receive an average of 0.5×10^6 to 1.0×10^6 molecules of RNA. For comparisons of sense, antisense, and dsRNA activities, injections were compared with equal masses of RNA (i.e., dsRNA at half the molar concentration of the single strands). Numbers of molecules injected per adult are given as rough approximations based on concentration of RNA in the injected material (estimated from ethidium bromide staining) and injection volume (estimated from visible displacement at the site of injection). A variability of several-fold in injection volume between individual animals is possible; however, such variability would not affect any of the conclusions drawn herein.

Methods for Analysis of Phenotypes

Inhibition of endogenous genes was generally assayed in a wild type genetic background (N2). Features analyzed included movement, feeding, hatching, body shape, sexual identity, and fertility. Inhibition with *gfp* [27] and *lacZ* activity was assessed using strain PD4251. This strain is a stable transgenic strain containing an integrated array (ccIs4251) made up of three plasmids: pSAK4 (*myo-3* promoter driving mitochondrially targeted GFP), pSAK2 (*myo-3* promoter driving a nuclear targeted GFP-LacZ fusion), and a *dpy-20* subclone[26] as a selectable marker. This strain produces GFP in all body muscles, with a combination of mitochondrial and nuclear localization. The two distinct compartments are easily distinguished in these cells, allowing a facile distinction between

cells expressing both, either, or neither of the original GFP constructs.

Gonadal injection was performed by inserting the microinjection needle into the gonadal syncitium of adults and expelling 20-100 pl of solution (see Reference 25). Body cavity injections followed a similar procedure, with needle insertion into regions of the head and tail beyond the positions of the two gonad arms. Injection into the cytoplasm of intestinal cells was another effective means of RNA delivery, and may be the least disruptive to the animal. After recovery and transfer to standard solid media, injected animals were transferred to fresh culture plates at 16 hour intervals. This yields a series of semi-synchronous cohorts in which it was straightforward to identify phenotypic differences. A characteristic temporal pattern of phenotypic severity is observed among progeny. First, there is a short "clearance" interval in which unaffected progeny are produced. These include impermeable fertilized eggs present at the time of injection. After the clearance period, individuals are produced which show the inhibitory phenotype. After injected animals have produced eggs for several days, gonads can in some cases "revert" to produce incompletely affected or phenotypically normal progeny.

Additional Description of the Results

Figure 1 shows genes used to study RNA-mediated genetic inhibition in *C. elegans*. Intron-exon structure for genes used to test RNA-mediated inhibition are shown (exons: filled boxes; introns: open boxes; 5' and 3' untranslated regions: shaded; sequence references are as follows: *unc-22* [9], *unc-54* [12], *fem-1* [14], and *hlh-1* [15]). These genes were chosen based on: (1) a defined molecular structure, (2) classical genetic data showing the nature of the null phenotype. Each segment tested for inhibitory effects is designated with the name of the gene followed by a single letter (e.g., *unc22C*). Segments derived from genomic DNA are shown above the gene, segments derived from cDNA are shown below the gene. The consequences of injecting double-stranded RNA segments for each of these genes is described in Table 1. dsRNA sequences from the coding region of each gene produced a phenotype resembling the null phenotype for that gene.

The effects of inhibitory RNA were analyzed in individual cells (Figure 2, panels A-H). These experiments were carried out in a reporter strain (called PD4251) expressing

two different reporter proteins: nuclear GFP-LacZ and mitochondrial GFP, both expressed in body muscle. The fluorescent nature of these reporter proteins allowed us to examine individual cells under the fluorescence microscope to determine the extent and generality of the observed inhibition of gene. ds-*unc22A* RNA was injected as a negative control. GFP expression in progeny of these injected animals was not affected. The GFP patterns of these progeny appeared identical to the parent strain, with prominent fluorescence in nuclei (the nuclear localized GFP-LacZ) and mitochondria (the mitochondrially targeted GFP): young larva (Figure 2A), adult (Figure 2B), and adult body wall at high magnification (Figure 2C).

In contrast, the progeny of animals injected with ds-*gfpG* RNA are affected (Figures 2D-F). Observable GFP fluorescence is completely absent in over 95% of the cells. Few active cells were seen in larvae (Figure 2D shows a larva with one active cell; uninjected controls show GFP activity in all 81 body wall muscle cells). Inhibition was not effective in all tissues: the entire vulval musculature expressed active GFP in an adult animal (Figure 2E). Rare GFP positive body wall muscle cells were also seen adult animals (two active cells are shown in Figure 2F). Inhibition was target specific (Figures 2G-I). Animals were injected with ds-*lacZL* RNA, which should affect the nuclear but not the mitochondrial reporter construct. In the animals derived from this injection, mitochondrial-targeted GFP appeared unaffected while the nuclear-targeted GFP-LacZ was absent from almost all cells (larva in Figure 2G). A typical adult lacked nuclear GFP-LacZ in almost all body-wall muscles but retained activity in vulval muscles (Figure 2H). Scale bars in Figure 2 are 20 μm.

The effects of double-stranded RNA corresponding to *mex-3* on levels of the endogenous mRNA was shown by *in situ* hybridization to embryos (Figure 3, panels A-D). The 1262 nt *mex-3* cDNA clone[20] was divided into two segments, *mex-3*A and *mex-3*B with a short (325 nt) overlap. Similar results were obtained in experiments with no overlap between inhibiting and probe segments. *mex-3*B antisense or dsRNA was injected into the gonads of adult animals, which were maintained under standard culture conditions for 24 hours before fixation and *in situ* hybridization (see Reference 5). The *mex-3*B dsRNA produced 100% embryonic arrest, while >90% of embryos from the antisense injections hatched. Antisense probes corresponding to *mex-3*A were used to

assay distribution of the endogenous *mex-3* mRNA (dark stain). Four-cell stage embryos were assayed; similar results were observed from the 1 to 8 cell stage and in the germline of injected adults. The negative control (the absence of hybridization probe) showed a lack of staining (Figure 3A). Embryos from uninjected parents showed a normal pattern of endogenous *mex-3* RNA (Figure 3B). The observed pattern of *mex-3* RNA was as previously described in Reference 20. Injection of purified *mex-3*B antisense RNA produced at most a modest effect: the resulting embryos retained *mex-3* mRNA, although levels may have been somewhat less than wild type (Figure 3C). In contrast, no *mex-3* RNA was detected in embryos from parents injected with dsRNA corresponding to *mex-3*B (Figure 3D). The scale of Figure 3 is such that each embryo is approximately 50 μm in length.

Gene-specific inhibitory activity by *unc-22A* RNA was measured as a function of RNA structure and concentration (Figure 4). Purified antisense and sense RNA from *unc22A* were injected individually or as an annealed mixture. "Control" was an unrelated dsRNA (*gfpG*). Injected animals were transferred to fresh culture plates 6 hours (columns labeled **1**), 15 hours (columns labeled **2**), 27 hours (columns labeled **3**), 41 hours (columns labeled **4**), and 56 hours (columns labeled **5**) after injection. Progeny grown to adulthood were scored for movement in their growth environment, then examined in 0.5 mM levamisole. The main graph indicates fractions in each behavioral class. Embryos in the uterus and already covered with an eggshell at the time of injection were not affected and, thus, are not included in the graph. The bottom-left diagram shows the genetically derived relationship between *unc-22* gene dosage and behavior based on analyses of *unc-22* heterozygotes and polyploids[8,3].

Figures 5 A-C show a process and examples of genetic inhibition following ingestion by *C. elegans* of dsRNAs from expressing bacteria. A general strategy for production of dsRNA is to clone segments of interest between flanking copies of the bacteriophage T7 promoter into a bacterial plasmid construct (Figure 5A). A bacterial strain (BL21/DE3)[28] expressing the T7 polymerase gene from an inducible (Lac) promoter was used as a host. A nuclease-resistant dsRNA was detected in lysates of transfected

bacteria. Comparable inhibition results were obtained with the two bacterial expression systems. A GFP-expressing *C. elegans* strain, PD4251 (see Figure 2), was fed on a native bacterial host. These animals show a uniformly high level of GFP fluorescence in body muscles (Figure 5B). PD4251 animals were also reared on a diet of bacteria expressing dsRNA corresponding to the coding region for *gfp*. Under the conditions of this experiment, 12% of these animals showed dramatic decreases in GFP (Figure 5C). As an alternative strategy, single copies of the T7 promoter were used to drive expression of an inverted-duplication for a segment of the target gene, either *unc-22* or *gfp*. This was comparably effective.

All references (e.g., books, articles, applications, and patents) cited in this specification are indicative of the level of skill in the art and their disclosures are incorporated herein in their entirety.

1. Izant, J. & Weintraub, H. *Cell* 36, 1007-1015 (1984).
2. Nellen, W. & Lichtenstein, C. *TIBS* 18, 419-423 (1993).
3. Fire, A., et al. *Development* 113, 503-514 (1991).
4. Guo, S. & Kemphues, K. *Cell* 81, 611-620 (1995).
5. Seydoux, G. & Fire, A. *Development* 120, 2823-2834 (1994).
6. Ausubel, F., et al. *Current Protocols in Molecular Biology*, John Wiley N.Y. (1990).
7. Brenner, S. *Genetics* 77, 71-94 (1974).
8. Moerman, D. & Baillie, D. *Genetics* 91, 95-104 (1979).
9. Benian, G., et al. *Genetics* 134, 1097-1104 (1993).
10. Proud, C. *TIBS* 20, 241-246 (1995).
11. Epstein H., et al. *J. Mol. Biol.* 90 291-300 (1974).
12. Karn, J., et al. *Proc. Natl. Acad. Sci. (U.S.A.)* 80, 4253-4257 (1983).
13. Doniach, T. & Hodgkin J.A. *Dev. Biol.* 106, 223-235 (1984).
14. Spence, A., et al. *Cell* 60, 981-990 (1990).
15. Krause, M., et al. *Cell* 63, 907-919 (1990).
16. Chen, L., et al. *Development*, 120, 1631-1641 (1994).
17. Dibb, N. J., et al. *J. Mol. Biol.* 205, 603-613 (1989).

18. Sulston, J., et al. *Dev. Biol.* 100, 64-119 (1983).

19. Sulston, J. & Horvitz, H. *Dev. Biol.* 82, 41-55 (1977).

20. Draper B.W., et al. *Cell* 87, 205-216 (1996).

21. Sulston, J., et al. *Nature* 356, 37-41 (1992).

22. Matzke, M. & Matzke, A. *Plant Physiol.* 107, 679-685 (1995).

23. Ratcliff, F., et al. *Science* 276, 1558-1560 (1997).

24. Latham, K. *Trends in Genetics* 12,134-138 (1996).

25. Mello, C. & Fire, A. *Methods in Cell Biology* 48, 451-482 (1995).

26. Clark, D., et al. *Mol. Gen. Genet.* 247, 367-378 (1995).

27. Chalfie, M., et al. *Science* 263, 802-805 (1994).

28. Studier, F., et al. *Methods in Enzymology* 185, 60-89 (1990).

29. Bowerman, B., et al. *Cell* 68, 1061-1075 (1992).

30. Mello, C.C., et al. *Cell* 70, 163-176 (1992).

31. van der Keyl, H., et al. *Develop. Dynamics* 201, 86-94 (1994).

32. Goeddel, D.V. *Gene Expression Technology*, Academic Press, 1990.

33. Kriegler, M. *Gene Transfer and Expression*, Stockton Press, 1990.

34. Murray, E.J. *Gene Transfer and Expression Protocols*, Humana Press, 1991.

Table 1. Effects of sense, antisense, and mixed RNAs on progeny of injected animals.

Gene and Segment		Size	Injected RNA	F1 Phenotype
unc-22			***unc-22* null mutants: strong twitchers**[7,8]	
unc22A[a]	exon 21-22	742	sense	wild type
			antisense	wild type
			sense+antisense	strong twitchers (100%)
unc22B	exon 27	1033	sense	wild type
			antisense	wild type
			sense+antisense	strong twitchers (100%)
unc22C	exon 21-22[b]	785	sense+antisense	strong twitchers (100%)
fem-1			***fem-1* null mutants: female (no sperm)**[13]	
fem1A	exon 10[c]	531	sense	hermaphrodite (98%)
			antisense	hermaphrodite (>98%)
			sense+antisense	female (72%)
fem1B	intron 8	556	sense+antisense	hermaphrodite (>98%)
unc-54			***unc-54* null mutants: paralyzed**[7,11]	
unc54A	exon 6	576	sense	wild type (100%)
			antisense	wild type (100%)
			sense+antisense	paralyzed (100%)[d]
unc54B	exon 6	651	sense	wild type (100%)
			antisense	wild type (100%)
			sense+antisense	paralyzed (100%)[d]
unc54C	exon 1-5	1015	sense+antisense	arrested embryos and larvae (100%)
unc54D	promoter	567	sense+antisense	wild type (100%)
unc54E	intron 1	369	sense+antisense	wild type (100%)
unc54F	intron 3	386	sense+antisense	wild type (100%)

Table 1 (continued).

Gene and Segment		Size	Injected RNA	F1 Phenotype
hlh-1			***hlh-1* null mutants: lumpy-dumpy larvae**[16]	
hlh1A	exons 1-6	1033	sense	wild type (<2% lpy-dpy)
			antisense	wild type (<2% lpy-dpy)
			sense+antisense	lpy-dpy larvae (>90%)[e]
hlh1B	exons 1-2	438	sense+antisense	lpy-dpy larvae (>80%)[e]
hlh1C	exons 4-6	299	sense+antisense	lpy-dpy larvae (>80%)[e]
hlh1D	intron 1	697	sense+antisense	wild type (<2% lpy-dpy)
myo-3 driven GFP transgenes [f]				
myo-3::NLS::gfp::lacZ			**makes nuclear GFP in body muscle**	
gfpG	exons 2-5	730	sense	nuclear GFP-LacZ pattern of parent strain
			antisense	nuclear GFP-LacZ pattern of parent strain
			sense+antisense	nuclear GFP-LacZ absent in 98% of cells
lacZL	exon 12-14	830	sense+antisense	nuclear GFP-LacZ absent in >95% of cells
myo-3::MtLS::gfp			**makes mitochondrial GFP in body muscle**	
gfpG	exons 2-5	730	sense	mitochondrial GFP pattern of parent strain
			antisense	mitochondrial GFP pattern of parent strain
			sense+antisense	mitochondrial GFP absent in 98% of cells
lacZL	exon 12-14	830	sense+antisense	mitochondrial GFP pattern of parent strain

Legend of Table 1

Each RNA was injected into 6-10 adult hermaphrodites ($0.5\text{-}1 \times 10^6$ molecules into each gonad arm). After 4-6 hours (to clear pre-fertilized eggs from the uterus) injected animals were transferred and eggs collected for 20-22 hours. Progeny phenotypes were scored upon hatching and subsequently at 12-24 hour intervals.

a: To obtain a semi-quantitative assessment of the relationship between RNA dose and phenotypic response, we injected each *unc22A* RNA preparation at a series of different concentrations. At the highest dose tested (3.6×10^6 molecules per gonad), the

individual sense and antisense *unc22A* preparations produced some visible twitching (1% and 11% of progeny respectively). Comparable doses of ds-*unc22A* RNA produced visible twitching in all progeny, while a 120-fold lower dose of ds-*unc22A* RNA produced visible twitching in 30% of progeny.

b: *unc22C* also carries the intervening intron (43 nt).

c: *fem1A* also carries a portion (131 nt) of intron 10.

d: Animals in the first affected broods (laid at 4-24 hours after injection) showed movement defects indistinguishable from those of null mutants in *unc-54*. A variable fraction of these animals (25-75%) failed to lay eggs (another phenotype of *unc-54* null mutants), while the remainder of the paralyzed animals were egg-laying positive. This may indicate partial inhibition of *unc-54* activity in vulval muscles. Animals from later broods frequently exhibit a distinct partial loss-of-function phenotype, with contractility in a subset of body wall muscles.

e: Phenotypes of *hlh-1* inhibitory RNA include arrested embryos and partially elongated L1 larvae (the *hlh-1* null phenotype) seen in virtually all progeny from injection of ds-*hlh1A* and about half of the affected animals from ds-*hlh1B* and ds-*hlh1C*) and a set of less severe defects (seen with the remainder of the animals from ds-*hlh1B* and ds-*hlh1C*). The less severe phenotypes are characteristic of partial loss of function for *hlh-1*.

f: The host for these injections, PD4251, expresses both mitochondrial GFP and nuclear GFP-LacZ. This allows simultaneous assay for inhibition of *gfp* (loss of all fluorescence) and *lacZ* (loss of nuclear fluorescence). The table describes scoring of animals as L1 larvae. ds-*gfpG* caused a loss of GFP in all but 0-3 of the 85 body muscles in these larvae. As these animals mature to adults, GFP activity was seen in 0-5 additional bodywall muscles and in the eight vulval muscles.

Table 2. Effect of injection point on genetic inhibition in injected animals and their progeny.

dsRNA	Site of injection	Injected animal phenotype	Progeny Phenotype
None	gonad or body cavity	no twitching	no twitching
None	gonad or body cavity	strong nuclear & mitochondrial GFP	strong nuclear & mitochondrial GFP
unc22B	Gonad	weak twitchers	strong twitchers
unc22B	Body Cavity Head	weak twitchers	strong twitchers
unc22B	Body Cavity Tail	weak twitchers	strong twitchers
gfpG	Gonad	lower nuclear & mitochondrial GFP	rare or absent nuclear & mitochondrial GFP
gfpG	Body Cavity Tail	lower nuclear & mitochondrial GFP	rare or absent nuclear & mitochondrial GFP
lacZL	Gonad	lower nuclear GFP	rare or absent nuclear GFP
lacZL	Body Cavity Tail	lower nuclear GFP	rare or absent nuclear GFP

Table 3. *C. elegans* can respond in a gene-specific manner to environmental dsRNA.

Bacterial Food	Movement	Germline Phenotype	GFP-Transgene Expression
BL21(DE3)	0% twitch	< 1% female	< 1% faint GFP
BL21(DE3) [*fem-1* dsRNA]	0% twitch	43% female	< 1% faint GFP
BL21(DE3) [*unc22* dsRNA]	85% twitch	< 1% female	< 1% faint GFP
BL21(DE3) [*gfp* dsRNA]	0% twitch	< 1% female	12% faint GFP

Table 4. Effects of bathing *C. elegans* in a solution containing dsRNA.

dsRNA	Biological Effect
unc-22	Twitching (similar to partial loss of *unc-22* function)
pos-1	Embryonic arrest (similar to loss of *pos-1* function)
sqt-3	Shortened body (Dpy) (similar to partial loss of *sqt-3* function)

In Table 2, gonad injections were carried out into the GFP reporter strain PD4251, which expresses both mitochondrial GFP and nuclear GFP-LacZ. This allowed simultaneous assay of inhibition with *gfp* (fainter overall fluorescence), *lacZ* (loss of nuclear fluorescence), and *unc-22* (twitching). Body cavity injections were carried out into the tail region, to minimize accidental injection of the gonad; equivalent results have been observed with injections into the anterior region of the body cavity. An equivalent set of injections was also performed into a single gonad arm. For all sites of injection, the entire progeny brood showed phenotypes identical to those described in Table 1. This included progeny produced from both injected and uninjected gonad arms. Injected animals were scored three days after recovery and showed somewhat less dramatic phenotypes than their progeny. This could in part be due to the persistence of products already present in the injected adult. After ds-*unc22B* injection, a fraction of the injected animals twitch weakly under standard growth conditions (10 out of 21 animals). Levamisole treatment led to twitching of 100% (21/21) of these animals. Similar effects were seen with ds-*unc22A*. Injections of ds-*gfpG* or ds-*lacZL* produced a dramatic decrease (but not elimination) of the corresponding GFP reporters. In some cases, isolated cells or parts of animals retained strong GFP activity. These were most frequently seen in the anterior region and around the vulva. Injections of ds-*gfpG* and ds-*lacZL* produced no twitching, while injections of ds-*unc22A* produced no change in GFP fluorescence pattern.

While the present invention has been described in connection with what is presently considered to be practical and preferred embodiments, it is understood that the invention is not to be limited or restricted to the disclosed embodiments but, on the contrary, is intended to cover various modifications and equivalent arrangements included within the spirit and scope of the appended claims.

Thus it is to be understood that variations in the described invention will be obvious to those skilled in the art without departing from the novel aspects of the present invention and such variations are intended to come within the scope of the present invention.

WE CLAIM:

1. A method to inhibit expression of a target gene in a cell comprising introduction of a ribonucleic acid (RNA) into the cell in an amount sufficient to inhibit expression of the target gene, wherein the RNA comprises a double-stranded structure with an identical nucleotide sequence as compared to a portion of the target gene.

2. The method of claim 1 in which the target gene is a cellular gene.

3. The method of claim 1 in which the target gene is an endogenous gene.

4. The method of claim 1 in which the target gene is a transgene.

5. The method of claim 1 in which the target gene is a viral gene.

6. The method of claim 1 in which the cell is from an animal.

7. The method of claim 1 in which the cell is from a plant.

8. The method of claim 6 in which the cell is from an invertebrate animal.

9. The method of claim 8 in which the cell is from a nematode.

10. The method of claim 1 in which the identical nucleotide sequence is at least 50 bases in length.

11. The method of claim 1 in which the target gene expression is inhibited by at least 10%.

12. The method of claim 1 in which the cell is present in an organism and inhibition of target gene expression demonstrates a loss-of function phenotype.

13. The method of claim 1 in which the RNA comprises one strand which is self-complementary .

14. The method of claim 1 in which the RNA comprises two separate complementary strands.

15. The method of claim 14 further comprising synthesis of the two complementary strands and initiation of RNA duplex formation outside the cell.

16. The method of claim 14 further comprising synthesis of the two complementary strands and initiation of RNA duplex formation inside the cell.

17. The method of claim 1 in which the cell is present in an organism, and the RNA is introduced within a body cavity of the organism and outside the cell.

18. The method of claim 1 in which the cell is present in an organism and the RNA is introduced by extracellular injection into the organism.

19. The method of claim 1 in which the cell is present in a first organism, and the RNA is introduced to the first organism by feeding a second, RNA-containing organism to the first organism.

20. The method of claim 19 in which the second organism is engineered to produce an RNA duplex.

21. The method of claim 1 in which an expression construct in the cell produces the RNA.

22. A method to inhibit expression of a target gene comprising:

(a) providing an organism containing a target cell, wherein the target cell contains the target gene and the target gene is expressed in the target cell;

(b) contacting a ribonucleic acid (RNA) with the organism, wherein the RNA is comprised of a double-stranded structure with duplexed ribonucleic acid strands and one of the strands is able to duplex with a portion of the target gene; and

(c) introducing the RNA into the target cell, thereby inhibiting expression of the target gene.

23. The method of claim 22 in which the organism is an animal.

24. The method of claim 22 in which the organism is a plant.

25. The method of claim 22 in which the organism is an invertebrate animal.

26. The method of claim 22 in which the organism is a nematode.

27. The method of claim 26 in which a formulation comprised of the RNA is applied on or adjacent to a plant, and disease associated with nematode infection of the plant is thereby reduced.

28. The method of claim 22 in which the identical nucleotide sequence is at least 50 nucleotides in length.

29. The method of claim 22 in which the expression of the target gene is inhibited by at least 10%.

30. The method of claim 22 in which the RNA is introduced within a body cavity of the organism and outside the target cell.

31. The method of claim 22 in which the RNA is introduced by extracellular injection into the organism.

32. The method of claim 22 in which the organism is contacted with the RNA by feeding the organism food containing the RNA.

33. The method of claim 32 in which a genetically-engineered host transcribing the RNA comprises the food.

34. The method of claim 22 in which at least one strand of the RNA is produced by transcription of an expression construct.

35. The method of claim 35 in which the organism is a nematode and the expression construct is contained in a plant, and disease associated with nematode infection of the plant is thereby reduced.

36. A cell containing an expression construct,

wherein the expression construct transcribes at least one ribonucleic acid (RNA) and the RNA forms a double-stranded structure with duplexed strands of ribonucleic acid,

whereby said cell contains the double-stranded RNA structure and is able to inhibit expression of a target gene when the RNA is contacted with an organism containing the target gene.

37. A transgenic animal containing said cell of claim 36.

38. A transgenic plant containing said cell of claim 36.

39. A kit comprising reagents for inhibiting expression of a target gene in a cell,

wherein said kit comprises a means for introduction of a ribonucleic acid (RNA) into the cell in an amount sufficient to inhibit expression of the target gene, and

wherein the RNA has a double-stranded structure with an identical nucleotide sequence as compared to a portion of the target gene.

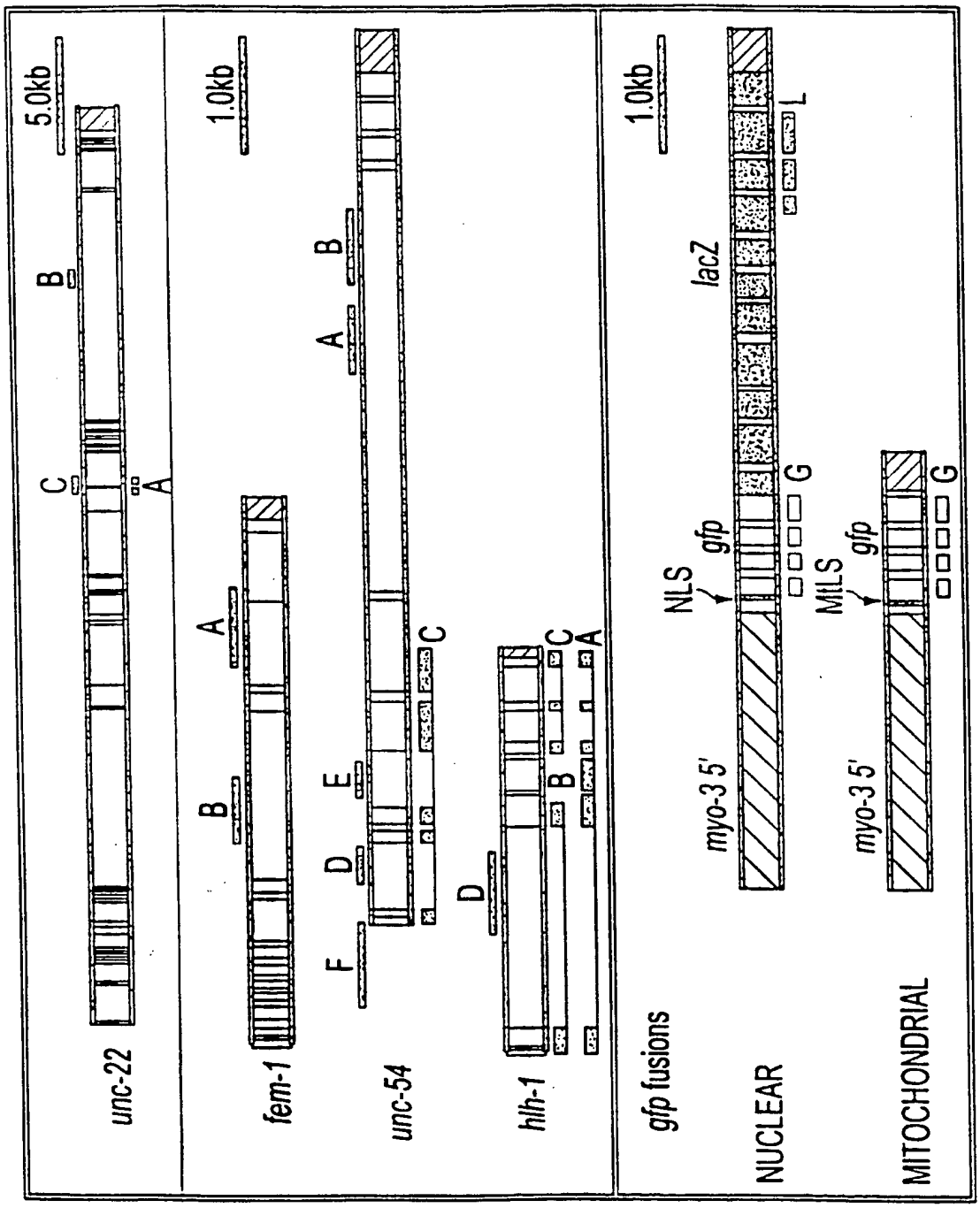
5.0kb
1.0kb
1.0kb
unc-22
fem-1
unc-54
hlh-1
gfp fusions
NUCLEAR
MITOCHONDRIAL
NLS
gfp
lacZ
MtLS
gfp
myo-3 5'
myo-3 5'
A
B
C
D
E
F
G
L

FIG. 1

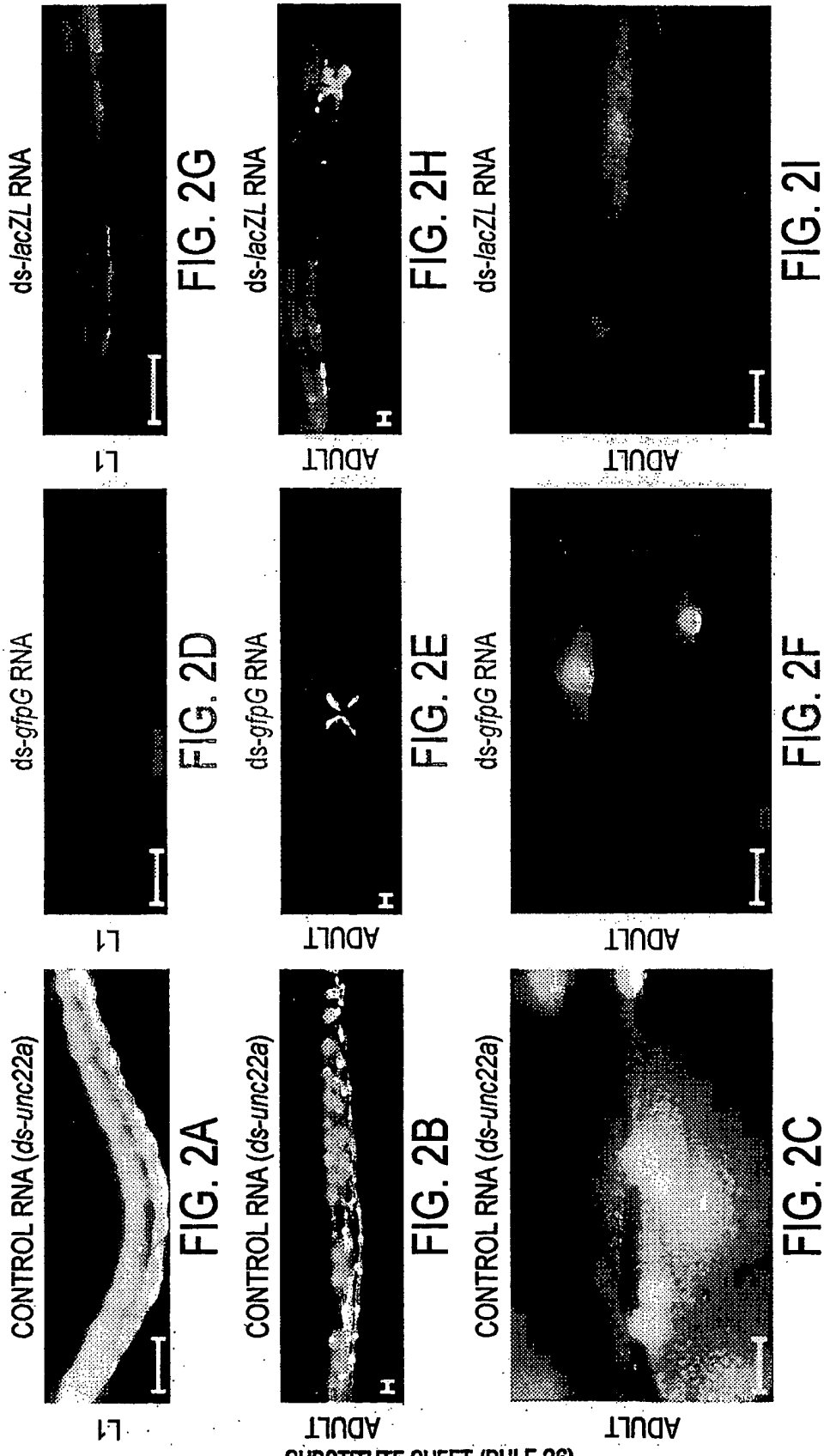

FIG. 2A FIG. 2B FIG. 2C

FIG. 2D FIG. 2E FIG. 2F

FIG. 2G FIG. 2H FIG. 2I

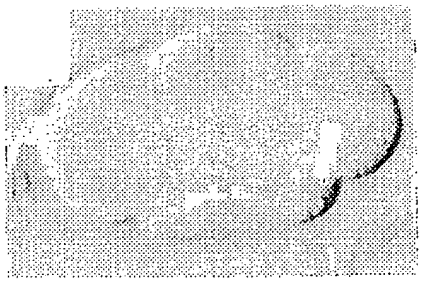

FIG. 3A

FIG. 3B

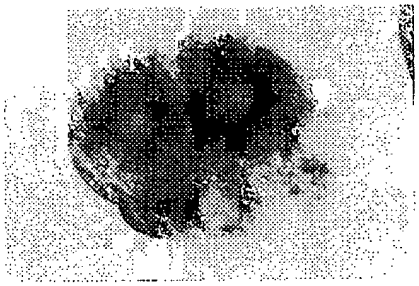

FIG. 3C

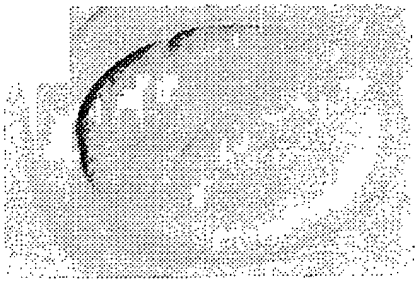

FIG. 3D

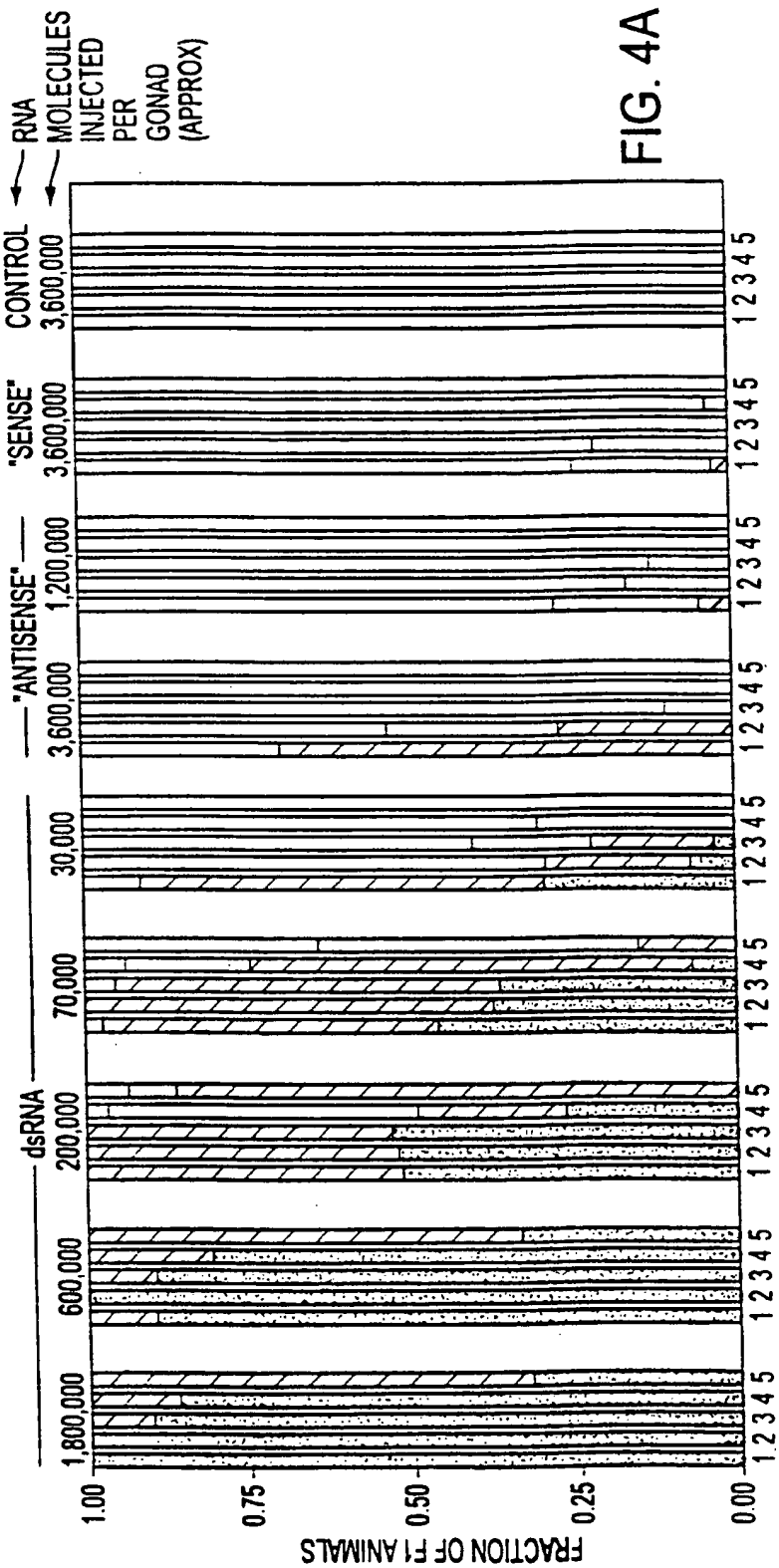
RNA MOLECULES INJECTED PER GONAD (APPROX)
CONTROL
"SENSE"
"ANTISENSE"
dsRNA
3,600,000
3,600,000
1,200,000
3,600,000
30,000
70,000
200,000
600,000
1,800,000
1 2 3 4 5
1.00
0.75
0.50
0.25
0.00
FRACTION OF F1 ANIMALS

FIG. 4A

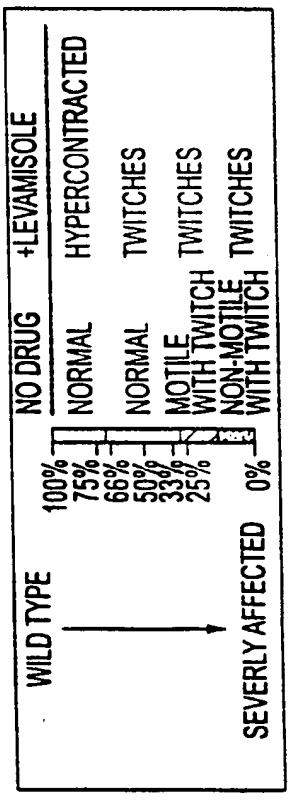
NO DRUG
+LEVAMISOLE
WILD TYPE
100%
75%
66%
50%
33%
25%
0%
NORMAL
HYPERCONTRACTED
NORMAL
TWITCHES
MOTILE WITH TWITCH
TWITCHES
NON-MOTILE WITH TWITCH
TWITCHES
SEVERLY AFFECTED

FIG. 4B

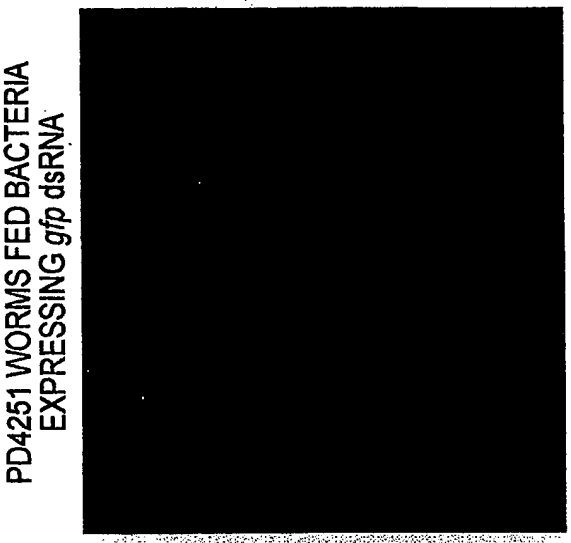

FIG. 5C

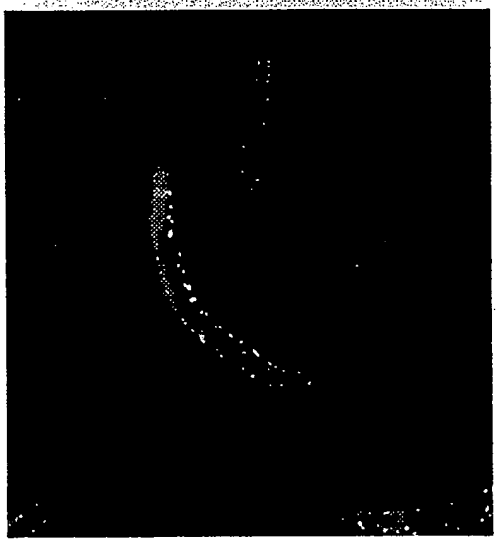

FIG. 5B

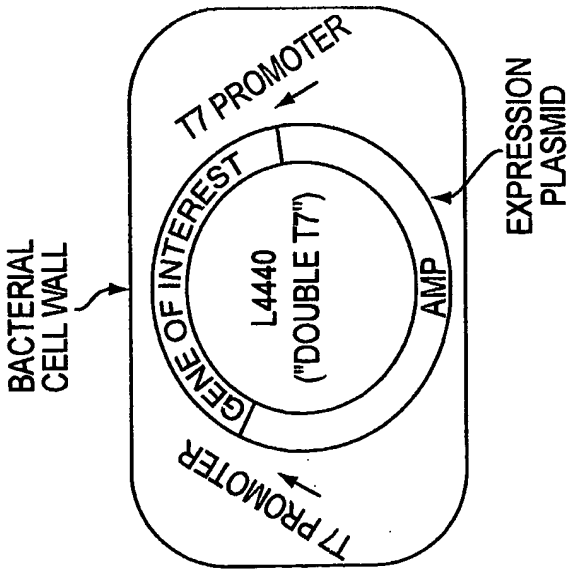

FIG. 5A

INTERNATIONAL SEARCH REPORT

Int ional Application No
PCT/US 98/27233

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12N15/11 C12N15/63 C12N15/82

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	RATCLIFF F ET AL: "A similarity between viral defense and gene silencing in plants" SCIENCE, vol. 276, no. 93, 6 June 1997, pages 1558-1560, XP002095874 see the whole document --- -/--	1

[X] Further documents are listed in the continuation of box C.

[ ] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
25 May 1999	10/06/1999

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	ANDRES, S

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Form PCT/ISA/210 (second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

Int tional Application No
PCT/US 98/27233

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
A	FIRE, A. ET AL.: "Production of antisense RNA leads to effective and specific inhibition of gene expression in C. elegans muscle" DEVELOPMENT (CAMBRIDGE, UK) (1991), 113(2), 503-14, XP002103600 cited in the application see page 508, right-hand column, paragraph 2 see page 509, right-hand column - page 511, right-hand column see page 512, 'Discussion' and figure 7 ---	1-39
A	MATZKE M A ET AL: "HOW AND WHY DO PLANTS INACTIVATE HOMOLOGOUS (TRANS)GENES?" PLANT PHYSIOLOGY, vol. 107, no. 3, 1 March 1995, pages 679-685, XP002021174 see page 680, left-hand column, paragraph 3 - right-hand column, paragraph 1 see page 682 ---	1
P,X	FIRE A ET AL: "Potent and specific genetic interference by double - stranded RNA in Caenorhabditis elegans" NATURE, (1998 FEB 19) 391 (6669) 806-11., XP002095876 cited in the application see the whole document ---	1-3,6, 8-12, 14-18, 21-23, 25,26, 28-31, 34,39
P,X	MONTGOMERY M K ET AL: "Double - stranded RNA as a mediator in sequence-specific genetic silencing and co - suppression" TRENDS IN GENETICS, (1998 JUL) 14 (7) 255-8., XP004124680 cited in the application see the whole document ---	1-4, 6-12, 14-18, 36-39
P,X	TIMMONS L ET AL: "Specific interference by ingested dsRNA" NATURE, (1998 OCT 29) 395 (6705) 854., XP002103601 cited in the application see the whole document -----	1-3,6, 8-12, 14-23, 25,26, 28-34, 36,39

1

INTERNATIONAL SEARCH REPORT

....ernational application No.
PCT/US 98/ 27233

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:
Remark: Although claim 35 and claims 1-6,8-23,25-26,34 (as far as in vivo methods practised on animals are concerned) are directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.

2. [ ] Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. [ ] As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

(19) Europäisches Patentamt
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(11) Publication number: **0 131 620 B1**

(12) **EUROPEAN PATENT SPECIFICATION published in accordance with Art. 158(3) EPC**

(45) Date of publication of patent specification: **21.08.91**

(51) Int. Cl.[5]: **C12N 15/84**, C12N 5/10, A01H 4/00, A01H 5/10

(21) Application number: **84900687.9**

(22) Date of filing: **16.01.84**

(86) International application number:
PCT/US84/00050

(87) International publication number:
WO 84/02920 (02.08.84 84/18)

(54) GENETICALLY TRANSFORMED PLANTS.

(30) Priority: **17.01.83 US 458402**

(43) Date of publication of application:
23.01.85 Bulletin 85/04

(45) Publication of the grant of the patent:
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(84) Designated Contracting States:
AT BE CH DE FR GB LI LU NL SE

(56) References cited:
EP-A- 0 116 718
WO-A-84/00466
WO-A-84/02919

N, Schell et al, Genetic Engineering to Biotechnology -The Critical Transition Edited by W.J. Whelan and Sandra Black Published by John Wiley and Sons Ltd. pages 41-52

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EP 0 131 620 B1

N, Otten et al, Mol. Gen. Genet. vol 183 pages 209-213 (1981)

N, Matzke et al, J. of Mol. Appl. Genet. vol no. 1 pages 39-49 (1981)

N, Leemans et al, J. of Mol. Appl. Genet. vol 1 no. 2 pages 149-164 (1981)

Plant Molecular Biology, vol.1, 1982, pages 265-276; Martinus Nijhoff/Dr. W. Junk Publishers, The Hague, NL G. Ooms et al.: "Studies on the structure of cointegrates between octopine and nopaline To-plasmids and their tumour-inducing properties."

Chemical Abstracts, vol. 101, no.3, July 1984, page 176, ref.no. 18452n; Columbus, Ohio, US J.D. Kemp et al.: "Transfer of a functional gene via the Ti plasmid."&Curr. Top. Plant Biochem. Physiol. Proc.Inaug. Plant Biochem. Physiol. Symp, 1982 (Pub. 1983), 1, 170-9

F. Ahmad et al.: "From Gene to Protein; Translation into Biotechnology", Miami Winter Symposia, vol. 19, 1982, page 514; Academic Press, New York, US P.V. Choudary et al.: "Studies on expression of the phaseolin gene of French bean seeds in sunflower plant cells."

Science, vol. 218, November 26, 1982, pages 854-859 L.W. Ream et al.: "Crown gall disease and prospects for genetic manipulation of plants."

G. Kahl et al.: "Molecular Biology of Plant Tumors", 1982, Chapter 21, pages 537-545; Academic Press, New York, US J. Leemans et aö.: "Ti plasmids and directed genetic engineering."

Chemical Abstracts, vol. 97, 1982, page 182, ref.no. 139517v; Columbus, Ohio, US G.M.S. Van Slogteren et al.: "The lysopine dehydrogenase gene used as a marker for the selection of octopine crown gall cells." & Plant Mol. Biol. 1982, 1(2), 133-42

Chemical Abstracts, vol. 99, 1983, page 182, ref. no. 100292j; Columbus, Ohio, US L. Comai et al.: "A new technique for genetic engineering of Agrobacterium Ti plasmid." & Plasmid 1983, 10 (1), 21-30

Stadler Symposim, vol. 13, 1981, University of Missouri, pages 39-51, US M. Chilton et al.: "Tailoring the Agrobacterium Ti plasmid as a vector for plant genetic engineering"

Proceedings of the National Academy of Sciences of the USA, vol. 80, no.15, August 1983, pages 4803-4807; Washington, US R.T. Fraley et al.: "Expression of bacterial genes in plant cells"

Chemical and Engineering News, June 22, 1981, pages 33-44 J.L. Fox.: "Plant molecular biology beginning to flourish."

Nature, vol.276, December 7, 1978, pages 633-634; MacMillan Journals Ltd. J.E. Beringer et al.: "Transfer of the drug-resistance transposon Tn5 to Rhizobium."

Chemical Abstracts, vol.87, 1977, page 312, ref. no. 180824z; Columbus, Ohio, US C-K. Tsai et al.: "Regeneration of plants from tobacco protoplasts and some factors affecting the plant differentiation." & Sci.Sin. (Engl.Ed.) 1977, 20(4), 458-68.

Ann.Ref.Genet., vol. 16, 1982, pages 357-384; Annual Rviews Inc. M.W. Bevan et al.: "T-DNA of the Agrobacterium Ti and Ri plasmids."

Newsweek, August 10, 1981, pages 54,55 "Gene splicing on the farm"

Chemical Abstracts, vol. 83, 1975, page 471, ref. no. 5347s; Columbus, Ohio, US D. Hess et al.: "Transformation experiments in higher plants. Prerequisites for the use of isolated protoplasts (isolation from mesophyll and callus cultures, uptake of proteins and DNA, and regeneration of whole plants). & Colloq.Int. C.N.R.S., September 11-15, 1972 (Pub.1973), 212, 343-51

Nature, vol. 293, September 24, 1981, no.5830, pages 265-270; MacMillan Journals Ltd. E.C. Cooking et al.: "Aspects of plant genetic manipulation."

Molecular Genetics of the Bacteriaplant Interaction, Symposium, August 1982, pages 223-227; Springer-Verlag, DE J. Hille et al.: "Onc- and virgenes located on the Ti-plasmid of Agrobacterium tumefaciens."

Proc.Natl.Akad.Sci.USA, vol. 78, no.7, July 1981, pages 4151-4155; US F. Yang et al.: "Revertant seedlings from crown gall tumors retain a portion of the bacterial Ti plasmid DNA sequences."

Gene, vol.14, 1981, pages 33-50; Elsevier/North-Holland Biomedical Press, NL G. Ooms et al.: "Crown gall plant tumors of abnormal morphology, induced by Agrobacterium tumefaciens carrying mutated octopine TI plasmids; analysis of T-DNA functions."

Nature, vol.277, January 11, 1979, pages 129-131; MacMillan Journals Ltd L. Marton et al.: "In vitro transformation of cultured cells from Nicotiana tabacum by Agrobacterium tumefaciens."

Description

Technical Field

This invention is in the fields of genetic engineering, plant biology, and bacteriology.

BACKGROUND ART

In the past decade, the science of genetic engineering has developed rapidly. A variety of processes are known for inserting a heterologous gene into bacteria, whereby the bacteria become capable of efficient expression of the inserted genes. Such processes normally involve the use of plasmids which may be cleaved at one or more selected cleavage sites by restriction endonucleases. Typically, a gene of interest is obtained by cleaving one piece of DNA, and the resulting DNA fragment is mixed with a fragment obtained by cleaving a vector such as a plasmid. The different strands of DNA are then connected ("ligated") to each other to form a reconstituted plasmid. See, for example, U.S. Patents 4,237,224 (Cohen and Boyer, 1980); 4,264,731 (Shine, 1981); 4,273,875 (Manis, 1981); 4,322,499 (Baxter et al, 1982), and 4,336,336 (Silhavy et al, 1982).

A variety of other reference works are available. Some of these works describe the natural process whereby DNA is transcribed into mRNA and mRNA is translated into protein, see, e.g., L. Stryer, Biochemistry, 2nd edition, p 559 et seq. (W. H. Freeman and Co., 1981); A. L. Lehninger, Biochemistry, 2nd edition, p. 853 et seq. (Worth Publ., 1975). Other works describe methods and products of genetic manipulation; see, e.g., T. Maniatis et al, Molecular Cloning, A Laboratory Manual (Cold Spring Harbor Labs, 1982); J.K. Setlow and A. Hollaender, Genetic Engineering, Principles and Methods (Plenum Press, 1979). Hereafter, all references will be cited in abbreviated form; a list of complete citations is included after the Examples.

Most of the genetic engineering work performed to date involves the insertion of genes into various types of cells, primarily bacteria such as E. coli, various other microorganisms such as yeast, and mammalian cells. However, many of the techniques and substances used for genetic engineering of animal cells and microorganisms are not directly applicable to genetic engineering involving plants.

As used herein, the term "plant" refers to a multicellular differentiated organism that is capable of photosynthesis, such as angiosperms and multicellular algae. This does not include microorganisms, such as bacteria, yeast, and fungi. The term "plant cell" includes any cell derived from a plant; this includes undifferentiated tissue such as callus or crown gall tumor, as well as plant seeds, propagules, pollen, or plant embryos.

Ti and Ri Plasmids

The tumor-inducing (Ti) plasmid of Agrobacterium tumefaciens has been proposed for use as a natural vector for introducing foreign genetic information into plant cells (Hernalsteen et al 1980; Rorsch and Schilperoort, 1978). Certain types of A. tumefaciens are capable of infecting a wide variety of plant cells, causing crown gall disease. The process of infection is not fully understood. At least part of the Ti plasmid enters the plant cell. Various metabolic alterations occur, and part of the Ti plasmid is inserted into the genome of the plant (presumably into the chromosomes). The part of the Ti plasmid that enters the plant genome is designated as "transferred DNA" (T-DNA). T-DNA is stably maintained in the plant DNA (Chilton et al, 1977; Yadev et al, 1980; Willmitzer et al, 1980; Otten et al, 1981).

Research by several laboratories has led to the characterization of several structural (i.e., protein coding) genes located in T-DNA (Garfinkel et al 1981; Leemans et al 1982), as well as other DNA sequences which appear to serve various other functions. For example, two sequences referred to as the "left border" and the "right border" appear to delineate T-DNA and may be involved in the process whereby T-DNA is transferred into plant chromosomes (Zambryski et al 1982).

A different species of Agrobacterium, A. rhizogenes, carries a "root-inducing" (Ri) plasmid which is similar to the Ti plasmid. Infection of a plant cell by A. rhizogenes causes hairy root disease. Like the Ti plasmid, a segment of DNA called "T-DNA" (also referred to by some researchers as "R-DNA") is transferred into the plant genome of an infected cell.

Various other bacteria are also reported to be capable of causing genetic transformation of plant cells, including A. rubi and certain bacteria of the genus Rhizobium which have been treated with a mutagenic agent. Hooykaas et al, at page 156 of Setlow and Holaender, 1979.

As used herein, the term "Ti plasmid" includes any plasmid (1) which is contained in a microorganism,

other than a virus, which is capable of causing genetic transformation of one or more types of plants or plant cells, and (2) which contains a segment of DNA which is inserted into a plant genome. This includes Ri plasmids.

As used herein, the term "T-DNA" refers to a segment of DNA in or from a Ti plasmid (1) which has been inserted into the genome of one or more types of plant cells, or (2) which is contained in a segment of DNA that is located between two sequences of bases which are capable of serving as T-DNA borders. As used herein, the terms "T-DNA border" and "border" are determined and applied empirically; these terms shall refer to a sequence of bases which appears at or near the end of a segment of DNA which is transferred from a Ti plasmid into a plant genome.

Despite the existing knowledge of T-DNA and Ti plasmids, a variety of obstacles exist to the use of these vectors for the introduction of foreign genes which are expressed in genetically modified plants. Such obstacles include:

1) the large size (approximately 200,000 base pairs) and resulting complexity of Ti plasmids preclude the use of standard recombinant DNA techniques to genetically modify and/or insert foreign genes into specific sites in the T-DNA. For example, there are no known unique restriction endonuclease cleavage sites in a Ti plasmid (Leemans et al, 1982).

2) the T-DNA, which is inserted into and expressed in plant cells, contains genes which are involved in the production of high levels of phytohormones in the transformed plant cells (Leemans et al 1982). The high levels of phytohormones interfere with the normal metabolic and regenerative process of the cells, and prevent the formation of phenotypically normal plants from the cells (Braun and Wood, 1976; Yang et al, 1980). Exceptions to this are rare cases where the T-DNA has undergone extensive spontaneous deletions in planta to eliminate those genes involved in phytohormone production. Under these conditions, normal plants are reported to be obtainable at low frequency (Otten et al, 1981). However, the T-DNA genes involved in phytohormone production could not be deleted prior to this invention, since they were very important in the identification and/or selection of transformed plant cells (Marton et al, 1979).

As described above, simple recombinant DNA techniques for introducing foreign genes into plasmids are not applicable to the large Ti plasmid. As a result, several indirect methods have been developed and are discussed below. The first reported use of the Ti plasmid as a vector was in model experiments in which bacterial transposons were inserted into T-DNA and subsequently introduced into plant cells. The bacterial transposons were reported to be stably maintained in the plant genome (Hernalsteens et al, 1980; Garfinkel et al 1981). However, in these cases the transformed tumor tissues were found to be incapable of regeneration into normal plants, and there was no reported evidence for the expression of bacterial genes in the plant cells. In addition, because the insertion of bacterial transposons is believed to be essentially random, a great deal of effort was required to identify and localize the position of the inserted DNA in these examples. Therefore, this approach is not likely to be useful to introduce desired genes in a predictable manner into plants.

Other researchers have reported the use of intermediate vectors which replicate in both E.coli and A. tumefaciens (Matzke and Chilton, 1981; Leemans et al 1981; Garfinkel et al, 1981). The intermediate vectors contain relatively small subfragments of the Ti plasmid which can be manipulated using standard recombinant DNA techniques. The subfragments can be modified by the deletion of specific sequences or by the insertion of foreign genes at specific sites. The intermediate vectors containing the modified T DNA subfragment are then introduced into A. tumefaciens by transformation or conjugation. Double recombination between the modified T-DNA fragment on the intermediate vector and its wild-type counterpart on the Ti plasmid results in the replacement of the wild-type copy with the modified fragment. Cells which contain the recombined Ti plasmids can be selected using appropriate antibiotics.

Various foreign DNA's have been inserted at specific sites in the T-DNA by this method and they have been reported to be stably transferred into plant cells (Matzke and Chilton, 1981, Leemans et al 1981, 1982). However, such foreign genes have not been reported to be capable of expression in plant cells, and the transformed plant cells remain incapable of regeneration into normal plants. Furthermore, in the procedure described above, it is preferred for a double crossover event to occur, in order to substitute the modified DNA fragment for the wild-type copy. A single crossover results in the formation of a co-integrate plasmid which contains two copies of the T-DNA subfragments. This duplication is undesirable in these methods since homologous recombination, which can occur in A. tumefaciens cells or in plant cells, can result in the loss of the inserted foreign gene(s).

A major disadvantage of the above methods is that the frequency of double recombination is quite low, about 10^{-4} to 10^{-9} (Leemans et al, 1981) and it requires extensive effort to identify and isolate the rare double-crossover recombinants. As a result, the number and types of experiments which can be performed using existing methods for genetically engineering the Ti plasmid is severely limited.

Other Means for Inserting DNA into Plant Cells

A variety of other methods have been reported for inserting DNA into plant cells. One such method involves the use of lipid vesicles, also called liposomes, to encapsulate one or more DNA molecules. The liposomes and their DNA contents may be taken up by plant cells; see, e.g., Lurquin, 1981. If the inserted DNA can be incorporated into the plant genome, replicated, and inherited, the plant cells will be transformed.

Other alternate techniques involve contacting plant cells with DNA which is complexed with either (a) polycationic substances, such as poly-L-ornithine (Davey et al, 1980), or (b) calcium phosphate (Krens et al, 1982). Using these techniques, all or part of a Ti plasmid has been reportedly inserted into plant cells, causing tumorigenic alteration of the plant cells.

Another method has been developed involving the fusion of bacteria, which contain desired plasmids, with plant cells. Such methods involve converting the bacteria into spheroplasts and converting the plant cells into protoplasts. Both of these methods remove the cell wall barrier from the bacterial and plant cells, using enzymic digestion. The two cell types can then be used together by exposure to chemical agents, such as polyethylene glycol. See Hasezawa et al, 1981.

However, all of the foregoing techniques suffer from one or more of the following problems:

1. transformation efficiencies reported to date have been very low;
2. only small DNA molecules can be inserted into plant cells;
3. only small numbers of DNA molecules can be inserted into plant cells; and/or,
4. a gene which is inserted into a plant cell will not be stably maintained by the plant cell unless it is incorporated into the genome of the plant cell, i.e., unless the gene is inserted into a chromosome or plasmid that replicates in the plant cell.

For these and possibly other reasons, no one has yet reported expression of a gene insertec into a plant cell by any of the foregoing techniques, except for the tumorigenic transformations noted above.

Prior to this invention, no satisfactory method existed for the creation and identification of genetically transformed plant cells which could be routinely regenerated into morphologically normal plants.

SUMMARY OF THE INVENTION

This invention provides a method for genetically transforming plant cells which comprises contacting plant cells, which are susceptible to generic transformation by Agrobacterium cells, with Agrobacterium tumefaciens cells containing a co-integrate Ti plasmid comprising a disarmed T-DNA region, which region comprises in sequence

(i) a left Agrobacterium T-DNA border sequence,

(ii) a chimeric selectable marker gene which functions in plant cells comprising a promoter which functions in plant cells, a structural coding sequence encoding a neomycin phosphotransferase and a 3' non-translated region encoding a polyadenylation signal, and

(iii) a right Agrobacterium T-DNA border sequence.

This invention also relates to a method for regenerating differentiated plants from the transformed plant cells, and to seeds obtained from the plants.

This invention involves a first plasmid, such as pMON120, which has certain desired characteristics described below. The chimeric selectable marker gene as defined in (ii) above is inserted into this plasmid to obtain a derivative plasmid, such as pMON128. For example, plasmid pMON128 contains a chimeric gene which expresses neomycin phosphotransferase II (NPT II), an enzyme which inactivates certain antibiotics. The chimeric gene is capable of expression in plant cells.

The derivative plasmid is inserted into a suitable microorganism, such as Agrobacterium tumefaciens cells which contain Ti plasmids. In the A. tumefaciens cells, some of the inserted plasmids recombine with Ti plasmids to form a co-integrate plasmid; this is due to a region of homology between the two plasmids. Only a single crossover event is required to create the desired co-integrate plasmid.

Because of the characteristics of the inserted plasmid of this invention, the resulting co-integrate Ti plasmid contains the chimeric gene and/or any other inserted gene within the T-DNA region of the co-integrate plasmid. The inserted gene(s) are surrounded by at least two T-DNA borders, at least one of which was inserted into the Ti plasmid by the crossover event. By means of appropriate antibiotics, A. tumefaciens cells which do not have co-integrate Ti plasmids with inserted genes are killed.

A. tumefaciens cells with co-integrate plasmids are co-cultured with plant cells, such as protoplasts, protoplast-derived cells, plant cuttings, or intact plants, under conditions which allow the co-integrate Ti plasmids, or portions thereof, to enter the plant cells. Once inside the plant cells, a portion of the Ti plasmid

which is surrounded by the two T-DNA borders is inserted by natural processes into the plant genome. This segment of DNA contains the chimeric gene and/or any other desired gene(s). Preferably, the segment of vector DNA which is inserted into the plant genome does not contain any genes which would render the plant cell incapable of being regenerated into a differentiated, morphologically-normal plant. The transformed plant cell(s) may be regenerated into a morphologically-normal plant which will pass the inserted gene to its descendants.

A variety of uses exist for plants transformed by the method of this invention. For example, a gene which codes for an enzyme which inactivates a herbicide may be inserted into a plant. This would make the plant and its descendants resistant to the herbicide. Alternately, a gene which codes for a desired mammalian polypeptide such as growth hormone, insulin, interferon, or somatostatin may be inserted into plants. The plants may be grown and harvested, and the polypeptide could be extracted from the plant tissue.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1 is a flow chart indicating the steps of this invention.

FIG. 2 represents the creation of pMON41, a plasmid used to construct pMON120.

FIG. 3 represents the creation of M-4, an M13-derived DNA used to construct pMON109.

FIG. 4 represents the creation of pMON54, a plasmid used to construct pMON109.

FIG. 5 represents the creation of pMON109, a plasmid used to construct pMON120.

FIG. 6 represents the creation of pMON113, a plasmid used to construct pMON120.

FIG. 7 represents the creation of plasmid pMON120, an intermediate vector with three restriction endonuclease cleavage sites which are suitable for the insertion of a desired gene.

FIG. 8 represents the creation of pMON128, an intermediate vector which was obtained by inserting a chimeric NOS-NPT II kanamycin-resistance gene into pMON120.

FIG. 9 represents the cointegration of pMON128 with a wild-type Ti plasmid by means of a single crossover event, thereby creating a co-integrate plasmid with multiple borders.

FIG. 10 indicates the co-integration of pMON128 with a disarmed Ti-plasmid, thereby creating a non-tumorigenic cointegrate plasmid.

FIG. 11 is a graph comparing growth of transformed cells and non-transformed cells on kanamycin-containing medium.

DETAILED DESCRIPTION OF THE INVENTION

In one preferred embodiment of this invention, a variety of chimeric genes were inserted into plant cells using the steps that are summarized on the flow chart of Figure 1. As shown on Figure 1, three preliminary plasmids were prepared. Those plasmids were designated as:

1. pMON41, which contained a right border from a nopaline-type Ti plasmid, and the 5' portion of a nopaline synthase (NOS) gene. The construction of plasmid pMON41 is described below and shown in Figure 2.
2. pMON109, which contained the 3' portion of a NOS gene, and a selectable marker gene (spc/str) which allowed for the selection of A. tumefaciens cells having co-integrate Ti plasmids with chimeric genes. The construction of plasmid pMON109 is described below and shown in Figures 3, 4, and 5.
3. pMON113, which contained a region of DNA with a sequence that is identical to the sequence within the T-DNA portion of an octopine-type Ti plasmid. This region was designated as the "left inside homology" (LIH) region. The construction of pMON113 is described below and shown in Figure 6.

After these plasmids were assembled, each plasmid was digested by appropriate endonucleases to obtain a desired fragment. Three fragments (one from each of the three plasmids) were assembled in a triple ligation to obtain the intermediate vector, plasmid pMON120, as shown in Figure 7.

Plasmid pMON120 plays a key role in the embodiment of this invention that is described in detail below. This plasmid has the following characteristics:

1. pMON120 has at least three unique restriction endonuclease cleavage sites (EcoRI, ClaI, and HindIII) which allow for the convenient insertion of any desired gene.
2. pMON120 will replicate within normal E. coli cells. However, it will not replicate within normal Agrobacterium cells unless it co-integrates with another plasmid, such as a Ti plasmid, which will replicate in Agrobacterium cells.
3. pMON120 carries a marker gene which codes for an enzyme which confers resistance to two antibiotics, spectinomycin (spc) and streptomycin (str). This gene, referred to as the spc/str gene, is

expressed in E. coli and in A. tumefaciens, but not in plant cells. pMON120 does not carry genes which code for resistance to ampicillin or tetracycline.

4. pMON120 carries a sequence which is homologous to a sequence within the T-DNA portion of an octopine-type Ti plasmid of A. tumefaciens. This sequence is referred to as the "left inside homology" (LIH) region. This region of homology promotes a crossover event whereby pMON120, or a derivative of pMON120 such as pMON128, forms a co-integrate with the Ti plasmid if the two plasmids exist inside the same A. tumefaciens cell. By definition, the "co-integrate" plasmid is formed by a single crossover event. It contains all DNA sequences that previously existed in either the Ti plasmid or the pMON120-derived plasmid.

5. pMON120 carries a nopaline-type T-DNA "right border," i.e., a sequence which is capable of acting as one end (designated by convention as the right border) of a T-DNA sequence which is transferred from a Ti plasmid and inserted into the chromosome of a plant cell during transformation of the cell by A. tumefaciens.

6. pMON120 carries a gene (including a promoter) which codes for the expression of an enzyme, nopaline synthase (NOS). Once introduced into a plant cell, the NOS enzyme catalyzes the production of nopaline, a type of opine. In most types of plants, opines are non-detrimental compounds which accumulate at low levels; the presence of nopaline can be readily detected in plant tissue (Otten and Schilperoort, 1978). Opine genes may serve as useful marker genes to confirm transformation, since opines do not normally exist in untransformed plant cells. If desired, the NOS gene in pMON120 may be rendered non-functional by a variety of techniques known to those skilled in the art. For example, a BamHI cleavage site exists within the coding portion of the NOS gene; a stop codon or other appropriate oligonucleotide sequence could be inserted into this site to prevent the translation of NOS.

7. The relative location of the various genes, cleavage sites, and other sequences in pMON120 is very important to the performance of this invention. The entire pMON120 plasmid, or its derivative plasmid such as pMON128, will be contained in the co-integrate Ti plasmid. However, only part of the co-integrate Ti plasmid (the modified T-DNA region) will be inserted into the plant genome. Therefore, only a part of the pMON120-derived plasmid will be inserted into the plant genome. This portion begins at the T-DNA border, and stretches in one direction only to the region of homology. In pMON120, the NOS scorable marker, the spc/str selectable marker, and the three insertion sites are within the portion of pMON120 that would be transferred into the plant genome. However, the pBR322-derived region next to the LIH, and the PvuI cleavage site, probably would not be transferred into the plant genome. Importantly, this arrangement of pMON120 and its derivatives prevents the transfer of more than one region of homology into the plant genome, as discussed below.

8. pMON120 is about 8 kilobases long. This is sufficiently small to allow it to accomplish all of the objectives of this invention. However, if desired, it may be made somewhat smaller by the deletion of one or more nucleotide sequences which are not essential, using methods which are known to those skilled in the art. Such a reduction in size might improve the efficiency or other characteristics of the plasmid when used for this invention or for other purposes, as may be determined by those skilled in the art.

It is recognized that a wide variety of intermediate vectors which differ from pMON120 in one or more respects may be prepared and utilized by those skilled in the art. For example, the NOS marker used for scoring transformed plant cells might be deleted, or replaced or supplemented by a different scorable or selectable marker. One such marker gene might comprise an antibiotic-resistance gene such as the NOS-NPT II-NOS chimeric gene described below. As another example, the spc/str marker gene used for selecting A. tumefaciens cells with co-integrate plasmids might be deleted, or replaced or supplemented by a different scorable or selectable marker that is expressed in Agrobacteria. As another example, a variety of T-DNA borders (such as a nopaline-type "left" border, or an octopinetype left or right border) might be utilized. Similarly, more than one border (such as two or more nopaline right borders, or one nopaline right border and one octopine right border) might be inserted into the intermediate vector, in the desired orientation; this may increase the frequency of insertion of the T-DNA into the plant genome, as may be determined by those skilled in the art. It is also possible to insert both left and right borders (of any type) into an intermediate vector. It is also possible to increase the length of the region of homology; this is likely to increase the frequency of the desired single crossover event (Leemans et al, 1981). It is also possible to select an appropriate region of homology from any type of desired plasmid, such as a nopaline or agropine Ti plasmid or an Ri plasmid; such regions will allow the intermediate vector to form a co-integrate with any desired plasmid.

Method of Creating pMON120

Plasmid pMON120 was constructed from fragments derived from 3 other plasmids. These three plasmids were designated as pMON41, pMON109, and pMON113. The construction of each of these three plasmids is summarized below; additional information is provided in the examples.

Plasmid pMON41 contributed a nopaline-type T-DNA right border and the 5' portion of a nopaline synthase (NOS) gene to pMON120. It was created by the following method.

A nopaline-type Ti plasmid, designated as the pTiT37 plasmid, may be digested with the HindIII endonuclease to produce a variety of fragments, including a 3.4 kb fragment which is designated as the HindIII-23 fragment. This fragment contains the entire NOS gene and the T-DNA right border. The Applicants inserted a HindIII-23 fragment into a plasmid, pBR327 (Soberon et al, 1980), which had been digested with HindIII. The resulting plasmid, designated as pMON38, was digested with both HindIII and BamHI. This produced a 2.3 kb fragment which contains the nopaline-type right border and the 5' portion of a NOS gene (including the promoter region, the 5' non-translated region, and part of the structural sequence). This 2.3 kb fragment was inserted into a pBR327 plasmid which had been digested with HindIII and BamHI. The resulting plasmid was designated as pMON41, as shown in Figure 2.

A variety of strains of A. tumefaciens are publicly available from the American Type Culture Collection (Rockville, MD); accession numbers are listed in any ATCC catalog. Each strain contains a Ti plasmid which is likely to be suitable for use in this invention, as may be determined through routine experimentation by those skilled in the art.

Plasmid pMON109 contributed a spc/str selectable marker gene and the 3' portion of a NOS gene to pMON120. It was created by the following method.

Plasmid pMON38 (described above and shown on Figure 2) was digested with RsaI, which creates blunt ends as shown:

5'-GTAC

CATG

A 1.1 kb fragment was isolated, and digested with BamHI to obtain fragments of 720 bp and 400 bp, each of which had one blunt Rsa end and a cohesive BamHI end. These fragments were added to double stranded DNA from a phage M13 mp8 (Messing and Vieira, 1982) which had been digested with SmaI (which creates blunt ends) and BamHI. The mixture was ligated, transformed into cells and plated for recombinant phage. Recombinant phage DNA's which contained the inserted 720 bp fragment were identified by the size of the BamHI-SmaI insert. One of those phage DNA's was designated as M-4, as shown in Figure 3. The 720 bp insert contained the 3' non-translated region (including the polyadenylation signal indicated in the figures by a heavy dot) of the NOS gene, as well as the 3' portion of the structural sequence of the NOS gene. The 720 bp insert is surrounded in M-4 by EcoRI and PstI cleavage sites, which were present in the M13 mp8 DNA.

A bacterial transposon, designated as Tn7, is known to contain the spc/str gene, mentioned previously. The Tn7 transposon also contains a gene which causes the host cell to be resistant to the antibiotic trimethoprim. The exact location and orientation of the spc/str gene and the trimethoprim-resistance gene in Tn7, are not known. The Tn7 transposon may be obtained from a variety of cell strains which are publicly available. A strain of A. tumefaciens was isolated in which the Tn7 transposon had been inserted into the Hind III-23 region of a pTiT37 plasmid. The modified pTiT37 plasmid was designated as pGV3106 (Hernalsteens et al, 1980).

Plasmid pGV3106 was digested with HindIII, and the fragments were shotgun-cloned into pBR327 plasmids which had been digested with HindIII. These plasmids were inserted into E. coli cells, and cells which were ampicillin-resistant (due to a pBR327 gene) and trimethoprim-resistant (due to a Tn7 gene) were selected. The plasmid obtained from one colony was designated as pMON31. This plasmid contained a 6kb HindIII insert. The insert contained the spc/str-resistance gene and trimethoprim-resistance gene from Tn7, and the 3' portion of a NOS gene (which came from the pTiT37 plasmid).

Plasmid pMON31 was reduced in size twice. The first reduction was performed by digesting the plasmid with EcoRI, diluting the mixture to remove an 850 bp fragment, and religating the large fragment. The resulting plasmid, designated as pMON53, was obtained from transformed cells selected by their resistance to ampicillin and streptomycin. Resistance to trimethoprim was not determined.

Plasmid pMON53 was further reduced in size by digesting the plasmid with ClaI, diluting the mixture to remove a 2 kb fragment, and religating the large fragment. The resulting 5.2 kb plasmid was designated as pMON54, as shown in Figure 4. This plasmid contains the spc/str gene.

Plasmid pMON54 was digested with EcoRI and PstI, and a 4.8 kb fragment containing the spc/str gene

was isolated. M-4 DNA was digested with EcoRI and PstI, and a 740 bp fragment containing the NCS 3' non-translated region was isolated. These fragments were ligated together to form pMON64. In order to be able to obtain the NOS 3' portion and the spc/str gene on a single EcoRI-BamHI fragment, the orientation of the spc/str gene was reversed by digesting pMON64 with ClaI and religating the mixture. Plasmids having the desired orientation were identified by cleavage using EcoRI and BamHI. These plasmids were designated as pMON109, as shown in Figure 5.

Plasmid pMON113 contributed a region of homology to pMON120 which allows pMON120 to form a co-integrate plasmid when present in A. tumefaciens along with a Ti plasmid. The region of homology was taken from an octopine-type Ti plasmid. In the Ti plasmid, it is located near the left T-DNA border, within the T-DNA portion of the Ti plasmid. This region of homology is designated as the "left inside homology" (LIH) region.

A region of homology may be derived from any type of plasmid capable of transforming plant cells, such as any Ti plasmid or any Ri plasmid. An intermediate vector can be designed which can form a co-integrate plasmid with whatever type of plasmid the region of homology was derived from.

In addition, it might not be necessary for the region of homology to be located within the T-DNA. For example, it may be possible for a region of homology to be derived from a segment of a Ti plasmid which contains a T-DNA border and a sequences of bases outside the T-DNA region. Indeed, if the intermediate vector contains two appropriate T-DNA borders, it might be possible for the region of homology to be located entirely outside of the T-DNA region.

The Applicants obtained an E. coli culture with a pBR-derived plasmid containing the Bam-8 fragment of an octopine-type Ti plasmid. The Bam-8 fragment, which is about 7.5 kb, contains the left border and the LIH region of the Ti plasmid (Willmitzer et al, 1982; DeGreve et al, 1981). The Bam-8 fragment was inserted into the plasmid pBR327, which had been digested with BamHI. The resulting plasmid was designated as pMON90, as shown in Figure 6.

Plasmid pMON90 was digested with BglII, and a 2.6 kb fragment which contains the LIH region but not the left border was purified. The 2.6 kb fragment was treated with Klenow polymerase to convert the cohesive ends into blunt ends, and the fragment was digested with HindIII to obtain a 1.6 kb fragment (the desired fragment) and a 1 kb fragment. Both fragments were mixed with a pBR322 plasmid which hac been digested with PvuII and HindIII. The mixture was ligated, and inserted into E. coli cells. The cells were selected for ampicillin resistance, and scored for the presence of a SmaI site which exists on the 1.6 kb fragment but not the 1 kb fragment. A colony having the desired plasmid was identified, and the plasmid from this colony was designated as pMON113, as indicated by Figure 6.

To assemble pMON120, three fragments had to be isolated. Plasmid pMON41 was digested with PvuI and BamI, and a 1.5 kb fragment containing a nopaline-type right border and the 5' portion of a NOS gene was isolated. Plasmid pMON109 was digested with BamHI and EcoRI, and a 3.4 kb fragment containing a spc/str gene and the 3' part of a NOS gene was isolated. Plasmid pMON113 was digested with PvuI and EcoRI, and a 3.1 kb fragment containing the LIH region was isolated.

The three fragments were mixed together and ligated to form pMON120, as shown on Figure 7. A culture of E. coli containing pMON120 has been deposited with the American Type Culture Collection. This culture has been assigned accession number 39263.

It is recognized that a variety of different methods could be used to create pMON120, or any similar intermediate vector. For example, instead of the triple ligation, it would have been possible to assemble two of the desired fragments in a plasmid, and insert the third fragment into the plasmid.

Method of Using pMON120

As mentioned previously, pMON120 has three unique cleavage sites (EcoRI, ClaI, and HindIII) which are suitable for the insertion of any desired gene. These cleavage sites are located in the portion of pMON120 that will be inserted into a plant genome, so the inserted gene also will be inserted into the plant genome.

A variety of chimeric genes which are capable of expressing bacterial and mammalian polypeptides in plant cells have been created by the Applicants. These chimeric genes are described in detail in a separate application entitled "Chimeric Genes Suitable for Expression in Plant Cells," WO 84/ 02913
Those chimeric genes are suitable for use in this invention. They may be inserted into pMON120 to create a derivative plasmid, which may be utilized as described below.

In one preferred embodiment of this invention, a chimeric gene was created which comprises the following DNA sequences:

1. a promoter region and a 5' non-translated region derived from a nopaline synthase (NOS) gene;
2. a structural sequence derived from a neomycin phosphotransferase II (NPT II) gene; and

3. a 3' non-translated region derived from a NOS gene.

This chimeric NOS-NPT II-NOS gene was isolated on a DNA fragment having EcoRI ends. This fragment was inserted into the EcoRI cleavage site of pMON120, and the resulting plasmids (having chimeric gene inserts with opposite orientations) were designated as pMON128 and pMON129, as shown in Figure 8. Plasmid pMON129 has two copies of the chimeric gene; this may be a useful feature in certain types of work. Either plasmid may be utilized to transform plant cells, in the following manner. A culture of E. coli containing pMON128 has been deposited with the American Type Culture Collection. This culture has been assigned accession number 39264.

Plasmid pMON128 is inserted into a microorganism which contains an octopine-type Ti plasmid (or other suitable plasmid). Suitable microorganisms include A. tumefaciens and A. rhizogenes which carry Ti or Ri plasmids. Other microorganisms which might also be useful for use in this invention include other species of Agrobacterium, as well as bacteria in the genus Rhizobia. The suitability of these cells, or of any other cells known at present or hereafter discovered or created, for use in this invention may be determined through routine experimentation by those skilled in the art.

The plasmid may be inserted into the microorganism by any desired method, such as transformation (i.e., contacting plasmids with cells that have been treated to increase their uptake of DNA) or conjugation with cells that contain the pMON128 or other plasmids.

The inserted plasmid (such as pMON128) has a region which is homologous to a sequence within the Ti plasmid. This "LIH" region of homology allows a single crossover event whereby pMON128 and an octopine-type Ti plasmid combine with each other to form a co-integrate plasmid. See, e.g., Stryer, supra, at p. 752-754. Normally, this will occur within the A. tumefaciens cell after pMON128 has been inserted into the cell. Alternately, the co-integrate plasmid may be created in a different type of cell or in vitro, and then inserted into an A. tumefaciens or other type of cell which can transfer the co-integrate plasmid into plant cells.

The inserted plasmid, such as pMON128, combines with the Ti plasmid in the manner represented by Figures 9 or 10, depending upon what type of Ti plasmid is involved.

In Figure 9, item 2 represents the T-DNA portion of an octopine-type Ti plasmid. Item 4 represents the inserted plasmid, such as pMON128. When these two plasmids co-exist in the same cell, a crossover event can occur which results in the creation of co-integrate plasmid 6.

Co-integrate plasmid 6 has one left border 8, and two right borders 10 and 12. The two right borders are designated herein as the "proximal" right border 10 (the right border closest to left border 8), and the "distal" right border 12 (the right border that is more distant from left border 8. Proximate right border 10 was carried by plasmid 4; the distal right border was contained on Ti plasmid 2 before co-integration.

A culture of A. tumefaciens GV3111 containing a co-integrate plasmid formed by pMON128 and wild-type Ti plasmid pTiB6S3 has been deposited with the American Type Culture Collection. This culture has been assigned accession number 39266.

When co-integrate Ti plasmid 6, shown in Figure 9, is inserted into a plant cell, either of two regions of DNA may enter the plant genome, T-DNA region 14 or T-DNA region 16.

T-DNA region 14 is bounded by left border 8 and proximate right border 10. Region 14 contains the chimeric gene and any other genes contained in plasmid 4, such as the spc/str selectable marker and the NOS scorable marker. However, region 14 does not contain any of the T-DNA genes which would cause crown gall disease or otherwise disrupt the metabolism or regenerative capacity of the plant cell.

T-DNA region 16 contains left border 8 and both right borders 10 and 12. This segment of T-DNA contains the chimeric gene and any other genes contained in plasmid 4. However, T-DNA region 16 also contains the T-DNA genes which are believed to cause crown gall disease.

Either of the foregoing T-DNA segments, Region 14 or Region 16, might be transferred to the plant DNA. This is presumed to occur at a 50-50 probability for any given T-DNA transfer. This is likely to lead to a mixture of transformed cells, some of which are tumorous and some or which are non-tumorous. It is possible to isolate and cultivate non-tumorous cells from the mixture, as described in the examples.

An alternate approach has also been developed which avoids the need for isolating tumorous from non-tumorous cells. Several mutant strains of A.tumefaciens have been isolated which are incapable of causing crown gall disease. Such strains are usually referred to as "disarmed" Ti plasmids. A Ti or Ri plasmid may be disarmed by one or more of the following types of mutations:

1. Removal or inactivation of one of the border regions. One such disarmed octopine Plasmid which has a left border but not a right border, is designated as pAL4421; this plasmid is contained in A. tumefaciens strain LBA4421 (Ooms et al, 1982; Garfinkel et al, 1981).

2. Removal or inactivation of the one or more of the "tumor morphology", genes, designated as the tmr and tms genes. See, e.g., Leemans et al, 1982.

Various other types of disarmed Ti plasmids may be prepared using methods known to those skilled in the art. See Matzke and Chilton, 1981; Leemans et al, 1981; Koekman et al, 1979.

Figure 10 represents an octopine-type Ti plasmid with a T-DNA region 22 which undergoes mutation to delete the tms and tmr genes and the right border. This results in a disarmed Ti plasmid with partial T-DNA region 24. When plasmid 26 (such as pMON128) is inserted into a cell that carries the disarmed Ti plasmid 24, a crossover event occurs which creates a co-integrate Ti plasmid with disarmed T-DNA region 28. The LIH region of homology is repeated in this Ti plasmid, but the disarmed Ti plasmid does not contain any oncogenic genes. Alternately, if only the right border had been deleted from T-DNA region 22, then the tms and tmr genes and the octopine synthase (OCS) gene would be contained in the co-integrate disarmed Ti plasmid; however, they would have been located outside of the T-DNA borders.

The disarmed co-integrate Ti plasmid is used to infect plant cells, and T-DNA region 28 enters the plant genome, as shown by transformed DNA 30. Plant cells transformed by disarmed T-DNA 28 have normal phytohormone metabolism, and normal capability to be regenerated into differentiated plants.

After pMON128 is inserted into A. tumefaciens cells, the desired crossover event will occur in a certain fraction of the cells. Cells which contain co-integrate plasmids (whether virulent or disarmed) may be easily selected from other cells in which the crossover did not occur, in the following manner. Plasmid pMON120 and its derivatives contain a marker gene (spc/str), which is expressed in A. tumefaciens. However, these plasmids do not replicate in A. tumefaciens. Therefore, the spc/str marker gene will not be replicated or stably inherited by A. tumefaciens unless the inserted plasmid combines with another plasmid that can replicate in A. tumefaciens. The most probable such combination, due to the region of homology, is the co-integrate formed with the Ti plasmid. A. tumefaciens cells which contain this co-integrate plasmid can readily be identified and selected by growth of the cells on medium containing either spc or str, or both.

The Ti plasmid 28, shown in Figure 10, contains two LIH regions. It is possible that co-integrate Ti plasmids will undergo a subsequent crossover event, wherein the two LIH regions will recombine. This event is undesirable, since it can lead to a deletion of the DNA between the LIH regions, including the chimeric gene. However, this is not likely to lead to serious difficulties, for two reasons. First, this event is likely to occur at a relatively low probability, such as about 10^{-2}. Second, plasmid pMON120 and its derivatives have been designed so that the selectable marker gene (spc/str) is located in the region of DNA that would be deleted by the crossover event. Therefore, the selective conditions that are used to identify and culture Agrobacteria cells containing co-integrate plasmids will also serve to kill the descendants of cells that undergo a subsequent crossover event which eliminates the chimeric gene from the Ti plasmid.

Only one of the LIH regions in the co-integrate Ti plasmid will be inserted into the plant genome, as shown in Figure 10. This important feature results from the design of pMON120, and it distinguishes this co-integrate plasmid from undesired co-integrate plasmids formed by the prior art. The LIH region which lies outside the T-DNA borders will not be inserted into the plant genome. This leads to at least two important advantages. First, the presence of two LIH regions inserted into the plant genome could result in crossover events which would lead to loss of the inserted genes in the transformed plant cells and their progeny. Second, the presence of two regions of DNA homology can significantly complicate efforts to analyze the DNA inserted into the plant genome (Matzke and Chilton, 1981).

After A. tumefaciens cells which contain the co-integrate Ti plasmids with the chimeric genes have been identified and isolated, the co-integrate plasmids (or portions thereof) must be inserted into the plant cells. Eventually, methods may be developed to perform this step directly. In the meantime, a method has been developed which may be used conveniently and with good results.

The plant cells to be transformed are contacted with enzymes which remove the cell walls. This converts the plant cells into protoplasts, which are viable cells surrounded by membranes. The enzymes are removed, and the protoplasts begin to regenerate cell wall material. At an appropriate time, the A. tumefaciens cells (which contain the co-integrate Ti plasmids with chimeric genes) are mixed with the plant protoplasts. The cells are co-cultivated for a period of time which allows the A. tumefaciens to infect the plant cells. After an appropriate co-cultivation period, the A. tumefaciens cells are killed, and the plant cells are propagated.

Plant cells which have been transformed (i.e., cells which have received DNA from the co-integrate Ti plasmids) and their descendants may be selected by a variety of methods, depending upon the type of gene(s) that were inserted into the plant genome. For example, certain genes may cause various antibiotics to be inactivated; such genes include the chimeric NOS-NPT II-NOS gene carried by pMON128. Such genes may serve as selectable markers; a group of cells may be cultured on medium containing the antibiotic which is inactivated by the chimeric gene product, and only those cells containing the selectable marker gene will survive.

A variety of genes may serve as scorable markers in plant cells. For example, pMON120 and its

derivative plasmids, such as pMON128, carry a nopaline synthase (NOS) gene which is expressed in plant cells. This gene codes for an enzyme which catalyzes the formation of nopaline. Nopaline is a non-detrimental compound which usually is accumulated at sow quantities in most types of plants; it can be easily detected by electrophoretic or chromatographic methods.

If a plant is transformed by a gene which creates a polypeptide that is difficult to detect, then the presence of a selectable marker gene (such as the NOS-NPT II-NOS chimeric gene) or a scorable marker gene (such as the NOS gene) in the transforming vector may assist in the identification and isolation of transformed cells.

This invention is suitable for use with a wide variety of plants, as may be determined through routine experimentation by those skilled in the art. For example, this invention is likely to be useful to transform cells from any type of plant which can be infected by bacteria from the genus Agrobacterium. It is believed that virtually all dicotyledonous plants, and certain monocots, can be infected by one or more strains of Agrobacterium. In addition, microorganisms of the genus Rhizobia are likely to be useful for carrying co-integrate plasmids of this invention, as may be determined by those skilled in the art. Such bacteria might be preferred for certain types of transformations or plant types.

Certain types of plant cells can be cultured in vitro and regenerated into differentiated plants using techniques known to those skilled in the art. Such plant types include potatoes, tomatoes, carrots, alfalfa and sunflowers. Research in in vitro plant culture techniques is progressing rapidly, and methods are likely to be developed to permit the regeneration of a much wider range of plants from cells cultured in vitro. Cells from any such plant with regenerative capacity are likely to be transformable by the in vitro co-cultivation method discussed previously, as may be determined through routine experimentation by those skilled in the art. Such transformed plant cells may be regenerated into differentiated plants using the procedures described in the examples.

The in vitro co-cultivation method offers certain advantages in the transformation of plants which are susceptible to in vitro culturing and regeneration. However, this invention is not limited to in vitro cell culture methods. For example, a variety of plant shoots and cuttings (including soybeans, carrots, and sunflowers) have been transformed by contact with A. tumefaciens cells carrying the co-integrate plasmids of this invention. It is also possible to regenerate virtually any type of plant from a cutting or shoot. Therefore, it may be possible to develop methods of transforming shoots or cuttings using virulent or preferably disarmed co-integrate plasmids of this invention or mixtures thereof, and subsequently regenerating the transformed shoots or cuttings into differentiated plants which pass the inserted genes to their progeny.

This invention may be useful for a wide variety of purposes. For example, certain bacterial enzymes, such as 5-enol pyruvyl shikimate-3-phosphoric acid synthase (EPSP synthase) are inactivated by certain herbicides; other enzymes, such as glutathione-S-transferase (GST), inactivate certain herbicides. Using Agrobacterium cells containing a co-integrate Ti plasmid having a T-DNA region which comprises, in addition to the chimeric selectable marker gene, a gene which will cause expression of such enzymes in plant cells, it may be possible to cause plants regenerated from such transformed plant cells to become resistant to one or more herbicides. This would allow the herbicide, which would normally kill the untransformed plant, to be applied to a field of transformed plants. The herbicide would serve as a weed-killer, leaving the transformed plants undamaged.

Alternatively, it may be possible to insert chimeric genes into plants which will cause the plants to create mammalian polypeptides, such as insulin, interferon, growth hormone, etc. At an appropriate time, the plants (or cultured plant tissue) would be harvested. Using a variety of processes which are known to those skilled in the art, the desired protein may be extracted from the harvested plant tissue.

An alternative use of this invention is to create plants with high content of desired substances, such as storage proteins or other proteins. For example, a plant might contain one or more copies of a gene which codes for a desirable protein. Additional copies of this gene may be inserted into the plant by means of this invention. Alternatively, the structural sequence of the gene might be inserted into a chimeric gene under the control of a different promoter which causes prolific transcription of the structural sequence.

As used herein, "a piece of DNA" includes plasmids, phages, DNA fragments, and polynucleotides, whether natural or synthetic.

As used herein, a "chimeric piece of DNA" is limited to a piece of DNA which contains at least two portions (i.e., two nucleotide sequences) that were derived from different and distinct pieces of DNA. For example, a chimeric piece of DNA cannot be created by merely deleting one or more portions of a naturally existing plasmid. A chimeric piece of DNA may be produced by a variety of methods, such as ligating two fragments from different plasmids together, or by synthesizing a polynucleotide wherein the sequence of bases was determined by analysis of the base sequences of two different plasmids.

As used herein, a chimeric piece of DNA is limited to DNA which has been assembled, synthesized, or

otherwise produced as a result of man-made efforts, and any piece of DNA which is replicated or otherwise derived therefrom. "Man-made efforts" include enzymatic, cellular, and other biological processes, if such processes occur under conditions which are caused, enhanced, or controlled by human effort or intervention; this excluses plasmids, phages, and polynucleotides which are created solely by natural processes. As used herein, the term "derived from" shall be construed broadly. Whenever used in a claim, the term "chimeric" shall be a material limitation.

As used herein, "foreign" DNA includes any DNA which is inserted into a pre-existing plant cell. A "foreign gene" is a gene which is inserted into a pre-existing plant cell.

As used herein, a "marker gene" is a gene which confers a phenotypically identifiable trait upon the host cell which allows transformed host cells to be distinguished from non-transformed cells. This includes screenable, scorable, and selectable markers.

As used herein, a "region of homology" refers to a sequence of bases in one plasmid which has sufficient correlation with a sequence of bases in a different plasmid to cause recombination of the plasmid to occur at a statistically determinable frequency. Preferably, such recombination should occur at a frequency which allows for the convenient selection of cells having combined plasmids, e.g., greater than 1 per 10^5 cells. This term is described more fully in a variety of publications, e.g., Leemans et al, 1981.

Those skilled in the art will recognize, or be able to ascertain using no more than routine experimentation, numerous equivalents to the specific embodiments of the invention discussed herein. Such equivalents are within the scope of this invention.

EXAMPLES

Example 1: Creation of Plasmid pMON41

A culture of E. coli, carrying a pBR325 plasmid (Bolivar, 1978) with the HindIII-23 fragment of pTiT37 (Hernalsteens, et al, 1980) inserted at the HindIII site, was obtained from Drs. M. Bevan and M.D. Chilton, Washington University, St. Louis, MO. Ten micrograms (ug) of the plasmid from this clone was digested with 10 units of HindIII (unless noted, all restriction endonucleases used in these constructions were purchased from New England Biolabs, Beverly, MA and used with buffers according to the supplier's instructions) for 1 hour at 37°C. The 3.4 kb HindIII-23 fragment was purified by adsorption on glass beads (Vogelstein and Gillespie, 1979) after separation from the other HindIII fragments by electrophoresis on a 0.8% agarose gel. The purified 3.4 kb HindIII fragment (1.0 ug) was mixed with 1.0 ug of plasmid pBR327 DNA (Soberon, et al, 1980) that had been digested with both HindIII (2 units, 1 hour, 37°C and calf alkaline phosphatase (CAP; 0.2 units, 1 hour, 37°C de-proteinized with phenol, ethanol precipitated, and resuspended in 10 ul of TE (10 mM Tris HCl, pH8, 1 mM EDTA). One unit of T4 DNA ligase (prepared by the method of Murray et al, 1979) was added to the fragment mixture. One unit is defined as the concentration sufficient to obtain greater than 90% circularization of one microgram of HindIII linearized pBR327 plasmid in 5 minutes at 22°C. The mixed fragments were contained in a total volume of 15 ul of 25 mM Tris-HCl pH8, 10 mM $MgCl_2$, 1 mM dithiothreitol, 200 uM spermidine HCl and 0.75 mM ATP (ligase buffer).

The mixture was incubated at 22°C for 3 hours and then mixed with E. coli C600 recA56 cells that were prepared for transformation by treatment with $CaCl_2$ (Maniatis et al, 1982). Following a period for expression of the ampicillin resistance determinant carried by the pBR327 vector, cells were spread on LB solid medium plates (Miller, 1972) containing ampicillin at 200 ug/ml. After incubation at 37°C for 16 hours, several hundred clones were obtained. Plasmid mini-preps (Ish-Horowicz and Burke, 1981) were performed on 24 of these colonies and aliquots of the plasmid DNA's obtained (0.1 ug) were digested with HindIII to demonstrate the presence of the 3.4 kb HindIII fragment. One plasmid demonstrated the expected structure and was designated pMON38. pMON38 DNA were prepared by Triton® X-100 lysis and CsCl gradient procedure (Davis et al, 1980).

Fifty ug of pMON38 DNA were digested with HindIII and BamHI (50 units each, 2 hours, 37°C and the 2.3 kb HindIII-BamHI fragment was purified as described above. The purified fragment (1 ug) was mixed with 1 ug of the 2.9 kb HindIII-BamHI fragment of the pBR327 vector purified as described above. Following ligation (T4 DNA ligase, 2 units) and transformation of E. coli cells as described above, fifty ampicillin-resistant colonies were obtained. DNAs from twelve plasmid mini-preps were digested with HindIII and BamHI to ascertain the presence of the 2.3 kb fragment. One plasmid of the correct structure was chosen and designated pMON41, as shown in Figure 2. A quantity of this DNA was prepared as described above.

Example 2: Creation of M13 Clone M-4

Thirty ug of plasmid pMON38 (described in Example 1) were digested with RsaI (30 units, 2 hours, 37°C and the 1100 bp RsaI fragment was purified after separation by agarose gel electrophoresis using the glass bead method described in the previous example. The purified 1100 bp RsaI-RsaI fragment (1 ug) was digested with 2 units of BamHI and the BamHI was inactivated by heating. This DNA was mixed with 0.2 ug of phage M13mp8RF DNA which had been previously digested with SmaI and BamHI (2 units each, 1 hour, 37°C and 0.2 units of calf alkaline phosphatase (CAP). Following ligation with 100 units of T4 DNA ligase, transformation of E. coli JM101 cells as described in the previous example, the transformed cells were mixed with soft agar and plated under conditions that allow the identification of recombinant phage (Messing and Vieira, 1982). Twelve recombinant phage producing cells were picked and RF plasmid mini-preps were obtained as described in the previous example. The RF DNAs were digested with BamHI and SmaI to prove the presence of the 720 bp RsaI-BamHI fragment. One of the recombinant RF DNAs carrying the correct fragment was designated M13 mp8 M-4. This procedure is represented in Figure 3. M-4 RF DNA was prepared using the procedures of Ish-Horowicz and Burke, 1981 and Colman et al, 1978.

Example 3: Construction of pMON109

Twenty ug of plasmid pGV3106 (Hernalsteens et a1 1980, prepared by the method of Currier and Nester 1976) was digested with HindIII (20 units, 2 hours, 37°C and mixed with 2 ug of HindIII-digested pBR327. Following ligation (T4 DNA ligase, 2 units) and transformation of E. coli cells as described above, one colony resistant to trimethoprim (100 ug/ml) and ampicillin was obtained. Digestion of plasmid DNA from this cell demonstrated the presence of a 6 kb HindIII fragment. This plasmid was designated pMON31.

Plasmid pMON31 from a mini-prep (0.5 ug) was digested with EcoRI (1 unit, 1 hour, 37°C) and the endonuclease was inactivated by heating (10 min, 70°C). The 8.5 kb plasmid fragment was re-circularized in a ligation reaction of 100 ul (T4 DNA ligase, 1 unit) and used to transform E. coli cells with selection for ampicillin and streptomycin (25 ug/ml) resistant colonies. Plasmid mini-prep DNA's from six clones were digested with EcoRI to ascertain loss of the 850 bp fragment. One plasmid lacking the 850 bp EcoRI fragment was designated pMON53. This plasmid was introduced into E. coli GM42 dam- cells (Bale et al, 1979) by transformation as described.

Plasmid pMON53 (0.5 ug) from a mini-prep prepared from dam- cells was digested with ClaI, and recircularized in dilute solution as described above. Following transformation of E. coli GM42 cells and selection for ampicillin and spectinomycin (50 ug/ml) resistant clones, fifty colonies were obtained. Digestion of plasmid mini-prep DNA's from six colonies showed that all lacked the 2 kb ClaI fragment. One of these plasmids was designated pMON54, as represented in Figure 4. Plasmid DNA was prepared as described in Example 1.

Plasmid pMON54 DNA (20 ug) was digested with EcoRI and PstI (20 units of each, 2 hours, 37°C) and the 4.8 kb fragment was purified from agarose gels using NA-45 membrane (Schleicher and Schuell, Keene, N.H.).

The purified 4.8kb fragment (0.5 ug) was mixed with 0.3 ug of a 740 bp EcoRI-PstI fragment obtained from M13mp8 M-4 RF DNA (described in Example 2) which was purified using NA-45 membrane. Following ligation (T4 DNA ligase, 2 units), transformation of E. coli GM42 dam- cells, and selection for spectinomycin resistant cells, twenty colonies were obtained. Plasmid mini-prep DNA's prepared from twelve of these clones were digested with PstI and EcoRI to demonstrate the presence of the 740 bp fragment. One plasmid carrying this fragment was designated pMON64. A quantity of this plasmid DNA was prepared as described in Example 1.

DNA (0.5 ug) of pMON64 was digested with ClaI (1 unit, 1 hour, 37°C), the ClaI was heat inactivated, and the fragments rejoined with T4 DNA ligase (1 unit). Following transformation and selection for spectinomycin resistant cells, plasmid mini-preps from twelve colonies were made. The DNA's were digested with BamHI and EcoRI to determine the orientation of the 2 kb ClaI fragment. Half of the clones contained the ClaI fragment in the inverse orientation of that in pMON64. One of these plasmids was designated pMON109, as represented in Figure 5. DNA was prepared as described in Example 1.

Example 4: Creation of plasmid pMON113

Plasmid pNW31C-8,29C (Thomashow et al, 1980) was obtained from Dr. S. Gelvin of Purdue University, West Lafayette, IN. This plasmid carries the pTiA6 7.5 kb Bam-8 fragment. The Bam-8 fragment was purified from 50 ug of BamHI-digested pNW31C-8,29C using NA-45 membrane as described in previous examples. The purified 7.5 kb Bam-8 fragment (1.0 ug) was mixed with 0.5 ug of pBR327 vector DNA which had been previously digested with both BamHI (2 units) and 0.2 units of calf alkaline phosphatase (CAP) for

1 hour at 37°C the mixture was deproteinized and resuspended as described in previous examples. The mixed fragments were treated with T4 ligase (2 units), used to transform E. coli C600 recA cells and ampicillin-resistant colonies were selected as described previously. Mini-preps to obtain plasmid DNA were performed on twelve of these clones. The DNA was digested with BamHI to demonstrate the presence of the pBR327 vector and 7.5 kb Bam-8 fragments. One plasmid demonstrating both fragments was designated pMON90. DNA was prepared as described in Example 1.

Twenty-five ug of pMON90 DNA were digested with BglII (25 units, 2 hours, 37°C and the 2.6 kb BglII fragment was purified using NA-45 membrane. To create blunt ends, the fragment (2 ug) was resuspended in 10 ul of 50 mM NaCl, 6.6 mM Tris-HCl pH8, 6.6 mM $MgCl_2$ and 0.5 mM dithiothreitol (Klenow Buffer). The 4 deoxy-nucleoside triphosphates (dATP, dTTP, dCTP, and dGTP) were added to a final concentration of 1 mM and one unit of E. coli large Klenow fragment of DNA polymerase I (New England Biolabs, Beverly, MA) was added. After incubation for 20 minutes at 22°C, the Klenow polymerase was heat inactivated and 10 units of HindIII was added. The HindIII digestion was carried out for 1 hour at 37°C and then the enzyme was heat inactivated. The HindIII-BglII (blunt) fragments (1 ug) were added to 0.25 ug of the 2.2 kb HindIII-PvuII fragment of pBR322 (Bolivar, et al, 1977) which had been generated by HindIII and PvuII digestion then treating with calf alkaline phosphatase as described in previous examples. After ligation using 100 units of T4 DNA ligase, transformation of E. coli LE392 cells and selection of ampicillin-resistant colonies as described in previous examples, nineteen colonies were obtained. Plasmid mini-preps were prepared from twelve colonies and digested with HindIII to determine the size of the recombinant plasmid and with SmaI to determine that the correct fragment had been inserted. One plasmid with the correct structure was designated pMON113, as shown in Figure 6. Plasmid DNA was prepared as described in Example 1.

Example 5: Creation of Plasmid pMON120

Twenty ug of plasmid pMON109 (described in Example 3) were digested with EcoRI and BamHI (20 units each, 2 hours, 37°C) and the 3.4 kb BamHI-EcoRI fragment was purified using NA-45 membrane as described in previous examples. Twenty ug of plasmid pMON41 (described in Example 1) were digested with BamHI and PvuI (20 units each, 2 hours, 37°C) and the 1.5 kb BamHI-PvuI fragment purified using NA-45 membrane as described in previous examples.

Twenty ug of pMON113 DNA (described in Example 4) were digested with PvuI and EcoRI (2 units each, 2 hr, 37°C) and the 3.1 kb PvuI-EcoRI fragment was purified using NA-45 membrane as above. To assemble plasmid pMON120, the 3.1 kb EcoRI-PvuI pMON113 fragment (1.5 ug) was mixed with 1.5 ug of the 3.4 kb EcoRI-BamHI fragment from pMON109. After treatment with T4 ligase (3 units) for 16 hours at 10°C, the ligase was inactivated by heating (10 minutes, 70°C and 5 units of BamHI was added. Digestion continued for 30 minutes at 37°C at which time the BamHI endonuclease was inactivated by heating as above. Next, 0.75 ug of the 1.5 kb PvuI-BamHI fragment from pMON41 was added along with T4 DNA ligase (2 units) and fresh ATP to 0.75 mM final concentration. The final ligase reaction was carried out for 4 hours at 22°C at which time the mixture was used to transform E. coli LE 392 cells with subsequent selection for spectinomycin resistant cells as described previously. Plasmid mini-preps from twelve out of several thousand colonies were screened for plasmids of approximately 8 kb in size containing single sites for BamHI and EcoRI. One plasmid showing the correct structure was designated pMON120, which is shown in Figure 7 with an alternate method of construction. pMON120 DNA was prepared as described in Example 1.

A culture of E. coli containing pMON120 has been deposited with the American Type Culture Collection. This culture has been assigned accession number 39263.

Example 6: Creation of Plasmids pMON128 and pMON129

Plasmid pMON75 (described in detail in a separate application entitled "Chimeric Genes Suitable for Expression in Plant Cells," previously cited) contains a chimeric NOS-NPT II-NOS gene. This plasmid (and pMON128, described below) may be digested by EcoRI and a 1.5 kb fragment may be purified which contains the NOS-NPT II-NOS gene.

Plasmid pMON120 was digested with EcoRI and treated with calf alkaline phosphatase. After phenol deproteinization and ethanol precipitation, the EcoRI-cleaved pMON120 linear DNA was mixed with 0.5 ug of the 1.5 kb EcoRI chimeric gene fragment from pMON75 or 76. The mixture was treated with 2 units of T4 DNA ligase for 1 hour at 22°C. After transformation of E. coli cells and selection of colonies resistant to spectinomycin (50 ug/ml), several thousand colonies appeared. Six of these were picked, grown, and plasmid mini-preps made. The plasmid DNA's were digested with EcoRI to check for the 1.5 kb chimeric

gene insert and with BamHI to determine the orientation of the insert. BamHI digestion showned that in pMON128 the chimeric gene was transcribed in the same direction as the intact nopaline synthase gene of pMON120. A culture of E. coli containing pMON128 has been deposited with the American Type Culture Collection. This culture has been assigned accession number 39264. The orientation of the insert in pMON129 was opposite that in pMON128; the appearance of an additional 1.5 kb BamHI fragment in digests of pMON129 showed that plasmid pMON129 carried a tandem duplication of the chimeric NOS-NPT II-NOS gene, as shown in Figure 8.

Example 7: Creation of Co-integrate Plasmid pMON128::pTiB6S3TraC

Plasmid pMON128 (described in Example 6) was transferred to a chloramphenicol resistant Agrobacterium tumefaciens strain GV3111 = C58Cl carrying Ti plasmid pTiB6S3tra^{C} (Leemans, et al, 1982) using a tri-parental plate mating procedure, as follows. 0.2 ml of a culture of E. coli carrying pMON128 was mixed with 0.2 ml of a culture of E. coli strain HB101 carrying a pRK2013 plasmid (Ditta, et al, 1980) and 0.2 m1 of GV3111 cells. The mixture of cells was cultured in Luria Broth (LB), spread on an LB plate, and incubated for 16 to 24 hours at 30°C to allow plasmid transfer and generation of co-integrate plasmids. The cells were resuspended in 3 ml of 10 mM $MgSO_4$ and 0.2 ml aliquot was then spread on an LB plate containing 25 ug/ml chloramphenicol and 100 ug/ml each of spectinomycin and streptomycin. After incubation for 48 hr at 30°C approximately 10 colonies were obtained. One colony was chosen and grown at 30°C in LB medium containing chloramphenicol, spectinomycin, and streptomycin at the concentrations given above.

A separate type of co-integrate plasmid for use in control experiments was prepared by inserting pMON120 into A. tumefaciens cells, and selecting for cells with co-integrate plasmids using spectinomycin and streptomycin, as described above. Like pMON120, these plasmids do not contain the chimeric NOS-NPT II-NOS gene.

Example 8: Solutions Used in Plant Cell Cultures

The following solutions were used by the Applicants:

		per liter
Enzyme mix:	Cellulysin	5 g
	Macerozyme	0.7 g
	Ampicillin	0.4 g
	KH_2PO_4	27.2 mg
	KNO_3	101 mg
	$CaCl_2$	1.48 g
	$MgSO_4.7H_2O$	246 mg
	KI	0.16 mg
	$CuSO_4.5H_2O$	0.025 mg
	Mannitol	110 g
MS9:	MS salts(see below)	4.3 g
	Sucrose	30.0 g
	B5 vitamins (see below)	1 ml
	Mannitol	90.0 g
	Phytohormones:	
	Benzyladenine (BA)	0.5 mg
	2,4-D	1 mg
MS-ES	MS salts	4.3 g
	Sucrose	30 g
	B5 Vitamins	1 ml
	Mannitol	30 g
	Carbenicillin	10 mg
	Phytohormones:	
	Indole acetic acid	0.1 mg

MSO:	MS salts	4.3 g
	Sucrose	30.0 g
	B5 vitamins	1 ml
Feeder plate medium:	MS salts	4.3 g
	Sucrose	30.0 g
	B5 vitamins	1 ml
	Mannitol	30.0 g
	Phytohormones:	
	BA	0.5 mg
Ms2C	MS salts	4.3 g
	Sucrose	30 g
	B5 vitamins	1 ml
	Phytohormones:	
	chlorophenoxyacetic acid	2 mg
MS104:	MS salts	4.3 g
	Sucrose	30.0 g
	B5 vitamins	1 ml
	Phytohormones:	
	BA	0.1 mg
	NAA	1 mg
MS11:	MS salts	4.3 g
	Sucrose	30.0 g
	B5 vitamins	1 ml
	Phytohormones:	
	Zeatin	1 mg
B5 Vitamin stock:	myo-inositol	100 g
	thiamine HCl	10 g
	nicotinic acid	1 g
	pyrodoxine HCl	1 g
Float rinse:	MS salts	0.43 g
	Sucrose	171.2 g
	PVP-40	40.0 g

MS salts are purchased pre-mixed as a dry powder from Gibco Laboratories, Grand Island, N.Y.

Example 9: Preparation of Protoplasts

Mitchell petunia plants were grown in growth chambers with two or three banks of fluorescent lamps and two banks of incandescent bulbs (about 5,000 lux). The temperature was maintained at a constant 21°C and the lights were on for 12 hours per day. Plants were grown in a 50/50 mix of Vermiculite and Pro-mix BX (Premier Brands Inc., Canada). Plants were watered once a day with Hoagland's nutrient solution. Tissue was taken from dark green plants with compact, bushy growth. Leaves were sterilized in a solution of 10% commercial bleach and a small amount of detergent or Tween 20 for 20 minutes with occasional agitation. Leaves were rinsed two or three times with sterile distilled water, Thin strips (about 1 mm) were cut from the leaves, perpendicular to the main rib. The strips were placed in the enzyme mix at a ratio of about 1 g tissue to 10 ml enzymes. The dishes were sealed with parafilm, and incubated in the dark or under low, indirect light while gently agitating continuously (e.g., 40 rpm on gyrotary shaker). Enzymic incubations generally were run overnight, about 16-20 hours.

The digestion mixture was sieved through a 68, 74, or 88 um screen to remove large debris and leaf

material. The filtrate was spun at 70-100 g for five minutes to pellet the protoplasts. The supernatant was decanted and the pellet was gently resuspended in float rinse solution. This suspension was poured into babcock bottles. The bottles were filled to 2 or 3 cm above the base of the neck. 1 ml of growth medium MS9 was carefully layered on top of the float rinse.

The Babcock bottles were balanced and centrifuged at 500 to 1000 rpm for 10 to 20 minutes. The protoplasts formed a compact band in the neck at the interface. The band was removed with a pipette, taking care not to pick up any excess float rinse. The protoplasts were diluted into MS9. At this point, the protoplasts were washed with MS9 or diluted for plating without washing.

Protoplasts were suspended in MS9 medium at 5 x 10^4 per ml, and plated into T-75 flasks, at 6 ml per flask. Flasks were incubated on a level surface with dim, indirect light or in the dark at 26-28°C. On the third day following the removal of the enzymes from the leaf tissue, MSO (medium which does not contain mannitol) was added to each flask, using an amount equal to one-half the original volume. The same amount of MSO is added again on day 4. This reduces the mannitol concentration to about 0.33 M after the first dilution, and about 0.25 M after the second dilution.

Example 10: Co-cultivation with Bacteria

On day 5 following protoplast isolation, five to seven day old tobacco suspension cultures (TXD cells) were diluted (if necessary) with MS2C medium to the point where 1 ml would spread easily over the surface of agar medium in a 100 x 15 mm petri plate .this is a 10 to 15% suspension (w/v).. The agar medium was obtained by mixing 0.8% agar with MS-ES medium, autoclaving the mixture, and cooling the mixture until it solidifies in the plate. One ml of the TXD suspension was spread over 25 ml of feeder plate medium. An 8.5 cm disc of Whatman #1 filter paper was laid over the TXD feeder cells and smoothed out. A 7 cm disc of the same paper was placed in the center of the larger one.

Separately, aliquots of a culture of A. tumefaciens cells (grown in yeast extract peptone medium) were added to the flasks which contained the plant cells. One set of aliquots contained cells with the pMON128::Ti co-integrate plasmids having chimeric NOS-NPT II-NOS genes. The other set of aliquots contained cells with the pMON120::Ti co-integrate plasmids, which do not have chimeric NOS-NPT II-NOS genes.

The bacteria were added to the flasks to a density of 10^8 cells/ml. 0.5 ml of the cell mixture was spread in a thin layer on the surface of the 7 cm filter paper disc. The plates were wrapped in parafilm or plastic bags and incubated under direct fluorescent lighting, no more than five plates in a stack.

Within seven days, colonies were discernable. Within 14 days, the 7 cm discs, with colonies adhering to them, were transferred to new MSO agar medium (without feeder cells) containing 500 ug/ml carbenicillin, as well as 50 ug/ml of kanamycin sulfate (Sigma, St. Louis, MO). Within two weeks, vigorously growing green colonies could be observed on the plates which contained plant cells that had been co-cultured with A. tumefaciens strains containing the pMON128 co-integrate NOS-NPT II plasmid. No transformed colonies were detected on plates which contained plant cells that had been co-cultured with A. tumefaciens strains containing the pMON120 co-integrate plasmid. The kanamycin resistant transformants are capable of sustained growth in culture medium containing kanamycin. Southern blotting experiments (as described in E. Southern, J. Mol. Biol. 98: 503 (1975) confirmed that these cells contain the chimeric NOS-NPT II gene.

Both sets of transformed cells (and a third set of cells which had been transformed in the same manner by a chimeric gene coding for the enzyme NPT type I) were assayed for resistance to kanamycin. The results are indicated in Figure 11.

Example 11: Regeneration of Transformed Plants

The transformed kanamycin-resistant colonies described in Example 10 contained both tumorous and non-tumorous cells, as described in Figure 9 and the related text. The following procedure was used to isolate non-tumorous transformed cells from tumorous transformed cells, and to regenerate differentiated plant tissue from the non-tumorous cells.

Colonies were grown on MS104 agar medium containing 30 ug/ml kanamycin sulfate and 500 ug/ml carbenicillin until they reached about 1 cm in diameter. Predominantly tumorous colonies appear a somewhat paler shade of green and are more loosely organized than predominantly non-tumorous colonies. Non-tumorous colonies were removed from the MS104 medium by tweezers and placed upon MS11 medium containing 30 ug/ml kanamycin and 500 ug/ml carbenicillin. As the colonies continued to grow, colonies that appeared pale green and loosely organized were removed and discarded.

MS11 medium contains zeatin, a phytohormone which induces shooting formation in non-tumorous

colonies. Several shoots were eventually observed sprouting from kanamycin-resistant colonies. These shoots may be grown to a desired size, and cut off by a sharp blade, and inserted into agar medium without phytohormones, such as MSO, where it may generate roots. If desired, the medium may be supplemented by napthalene acetic acid to induce rooting. The plants may be grown to a desired size in the agar medium, and then transferred into soil. If properly cultivated, such plants will grow to maturity and generate seed. The acquired trait will be inherited by progeny according to classic Mendelian genetics.

References:

A. Bale et al, Mut. Res. 59: 157 (1979)
F. Bolivar, Gene 4: 121 (1978)
A. Braun and H. Wood, Proc. Natl. Acad. Sci. USA 73: 496 (1976)
M. D. Chilton et al , Cell 11: 263 (1977)
A. Colman et al, Eur. J. Biochem 91: 303-310 (1978)
T. Currier and E. Nester, J. Bact. 126: 157 (1976)
M. Davey et al, Plant Sci. Lett. 18: 307 (1980)
R. W. Davis et al, Advanced Bacterial Genetics Cold Spring Harbor Laboratory, New York, (1980)
H. De Greve et al, Plasmid 6: 235 (1981)
G. Ditta et al, Proc. Natl. Acad. Sci. USA 77: 7347 (1980)
D. Garfinkel et al, Cell 27: 143 (1981)
S. Hasezawa et al, Mol. Gen. Genet. 182: 206 (1981)
J. Hernalsteens et al, Nature 287: 654 (1980)
D. Ish-Horowicz and J. F. Burke, Nucleic Acids Res. 9: 2989-2998 (1981)
B. Koekman et al, J. Bacteriol. 141: 129 (1979)
F. Krens et al, Nature 296: 72 (1982)
J. Leemans et al, J. Mol. Appl. Genet. 1: 149 (1981)
J. Leemans et al, The EMBO J. 1: 147 (1982)
A. L. Lehninger, Biochemistry, 2nd ed. (Worth Publ., 1975)
P. Lurquin, Nucleic Acids. Res. 6: 3773 (1979)
T. Maniatis et al, Molecular Cloning, A Laboratory Manual (Cold Spring Harbor Labs, 1982)
L. Marton et al, Nature 277: 129 (1979)
T. Matzke and M-D Chilton, J. Mol. Appl. Genet. 1: 39 (1981)
J. Messing et al, Nucleic Acids Res. 9: 309 (1981)
J. Messing and J. Vieira, Gene 19: 269-276 (1982)
J. Miller, Experiments in Molecular Genetics, Cold Spring Harbor Laboratory, N.Y. (1972)
N. Murray et al, J. Mol. Biol. 132: 493 (1979)
G. Ooms et al, Plasmid 7: 15 (1982)
T. Otten and R. Schilperoort, Biochim. Biophys Acta 527: 497 (1978)
L. Otten, Mol. Gen. Genet. 183: 209 (1981)
R. Roberts, Nucleic Acids Res. 10: r117 (1982)
A. Rorsch and R. Schilperoort, Genetic Engineering, 189 Elsevier/North Holland, N.Y. (1978)
J. K. Setlow and A. Hollaender, Genetic Engineering, Principles and Methods (Plenum Press 1979)
X. Soberon et al, Gene 9: 287 (1980)
L. Stryer, Biochemistry, 2nd. ed. (W. H. Freeman and Co., 1981)
M. Thomashow et al, Cell 19: 729 (1980)
B. Vogelstein and D. Gillespie, Proc. Natl. Acad. Sci.: 615-619 (1979)
L. Willmitzer et al, Nature 287: 359 (1980)
L. Willmitzer et al, The EMBO J 1: 139 (1982)
N. Yadev et al, Nature 287: 1 (1980)
F. Yang et al, Mol. Gen. Genet. 177: 707 (1980)
P. Zambryski et al, J. Mol. Appl. Genet. 1: 361 (1982)

Claims

1. A method for genetically transforming plant cells which comprises, contacting plant cells, which are susceptible to genetic transformation by Agrobacterium cells, with Agrobacterium tumefaciens cells containing a co-integrate Ti plasmid comprising a disarmed T-DNA region which region comprises in sequence

(i) a left Agrobacterium T-DNA border sequence,
(ii) a chimeric selectable marker gene which functions in plant cells comprising a promoter which functions in plant cells, a structural coding sequence encoding a neomycin phosphotransferase and a 3' non-translated region encoding a polyadenylation signal, and
(iii) a right Agrobacterium T-DNA border sequence.

2. A method of Claim 1 in which the left T-DNA border is from an octopine Ti plasmid.

3. A method of Claim 2 in which the right T-DNA border is from a nopaline Ti plasmid.

4. A method of Claim 3 in which the left T-DNA border is from pTi B6S3 octopine Ti plasmid and the right T-DNA border is from pTi T37 nopaline Ti plasmid.

5. A method of Claim 1 further comprising regenerating said transformed plant cells into a differentiated plant.

6. A method of Claim 5 in which the transformed plant cells are cultured in a nutrient medium containing an aminoglycoside antibiotic.

7. A method of Claim 6 in which the aminoglycoside antibiotic is kanamycin.

8. A seed obtained from a plant produced by the method of any of Claims 5 to 7.

Revendications

1. Procédé pour transformer génétiquement des cellules végétales qui comprend la mise en contact des cellules végétales qui sont susceptibles de transformation génétique par des cellules d'Agrobacterium, avec des cellules d'Agrobacterium tumefaciens contenant un plasmide Ti co-intégré comprenant une région d'ADN-T désarmé , laquelle région comprend successivement :
(i) une séquence de frontière gauche d'ADN-T d'Agrobacterium,
(ii) un gène marqueur chimérique sélectionnable, qui fonctionne dans les cellules végétales, comprenant un promoteur qui fonctionne dans les cellules végétales, une séquence de codage structurale codant une néomycine phosphotransférase et une région non traduite 3' codant un signal de polyadénylation, et
(iii) une séquence frontière droite d'ADN-T d'Agrobacterium.

2. Procédé selon la revendication 1, dans lequel la frontière gauche de l'ADN-T provient d'un plasmide Ti d'octopine.

3. Procédé selon la revendication 2, dans lequel la frontière droite de l'ADN-T provient d'un plasmide Ti de nopaline.

4. Procédé selon la revendication 3, dans lequel la frontière gauche de l'ADN-T provient du plasmide Ti de l'octopine pTiB6S3 , la frontière droite de l'ADN-T provient du plasmide Ti de la nopaline de pTi T37.

5. Procédé selon la revendication 1, comprenant en outre la régénération de ces cellules végétales transformées en une plante différenciée.

6. Procédé selon la revendication 5, dans lequel les cellules végétales transformées sont cultivées dans un milieu nutritif contenant un antibiotique d'aminoglycoside.

7. Procédé selon la revendication 6, dans lequel l'antibiotique d'aminoglycoside est la kanamycine.

8. Graine obtenue à partir d'une plante produite par le procédé selon l'une quelconque des revendications 5 à 7.

Patentansprüche

1. Verfahren zum genetischen Transformieren von Pflanzenzellen, das das Inberührungbringen von Pflanzenzellen, die zur genetischen Transformation durch Agrobacterium-Zellen geeignet sind, mit Agrobacterium tumefaciens-Zellen, die ein co-integrales Ti-Plasmid umfassend einen entschärften T-DNA-Bereich enthalten, welcher Bereich in Sequenz
   (i) eine linke Agrobacterium-T-DNA-Grenzsequenz
   (ii) ein chimäres selektierbares Markergen, das in Pflanzenzellen wirkt, umfassend einen Promotor, der in Pflanzellenzellen wirkt, eine Strukturkodiersequenz, die eine Neomycinphosphotransferase kodiert, und einen 3'-nicht-translatierten Bereich, der ein Polyadenylierungssignal kodiert, und
   (iii) eine rechte Agrobacterium-T-DNA-Grenzsequenz aufweist, umfaßt.

2. Verfahren nach Anspruch 1, in dem die linke T-DNA-Grenze von einem Octopin-Ti-Plasmid ist.

3. Verfahren nach Anspruch 2, in dem die rechte T-DNA-Grenze von einem Nopalin-Ti-Plasmid ist.

4. Verfahren nach Anspruch 3, in dem die linke T-DNA-Grenze von pTi B6S3 Octopin-Ti-Plasmid und die rechte T-DNA-Grenze von pTi T37 Nopalin-Ti-Plasmid ist.

5. Verfahren nach Anspruch 1, das weiters das Regenerieren der transformierten Pflanzenzellen in eine differenzierte Pflanze umfaßt.

6. Verfahren nach Anspruch 5, in dem die transformierten Pflanzenzellen in einem Nährmedium gezüchtet werden, das ein Aminoglykosidantibiotikum enthält.

7. Verfahren nach Anspruch 6, in dem das Aminoglykosidantibiotikum Kanamycin ist.

8. Samen, erhalten aus einer Pflanze, hergestellt durch das Verfahren nach einem der Ansprüche 5 bis 7.

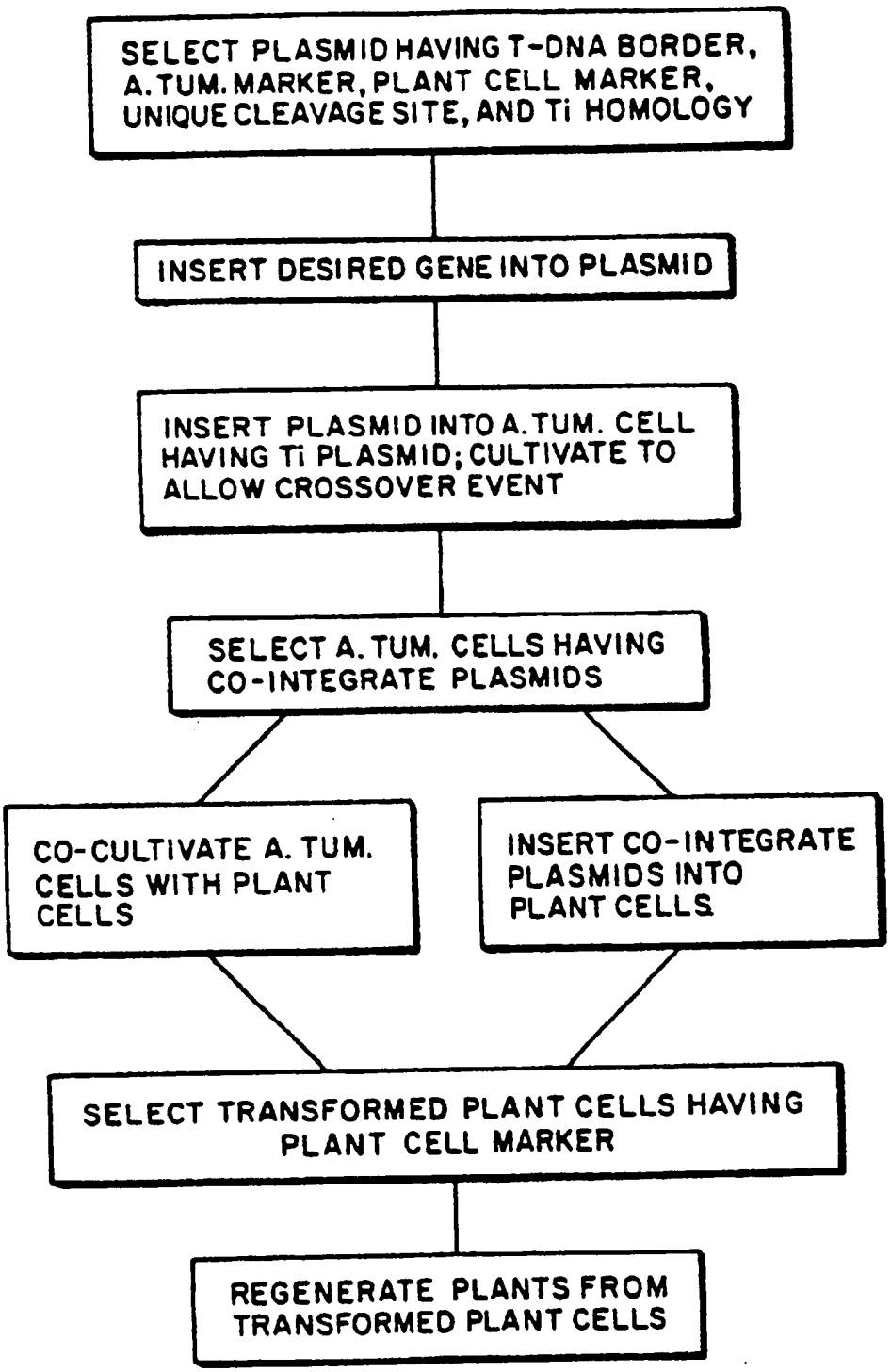
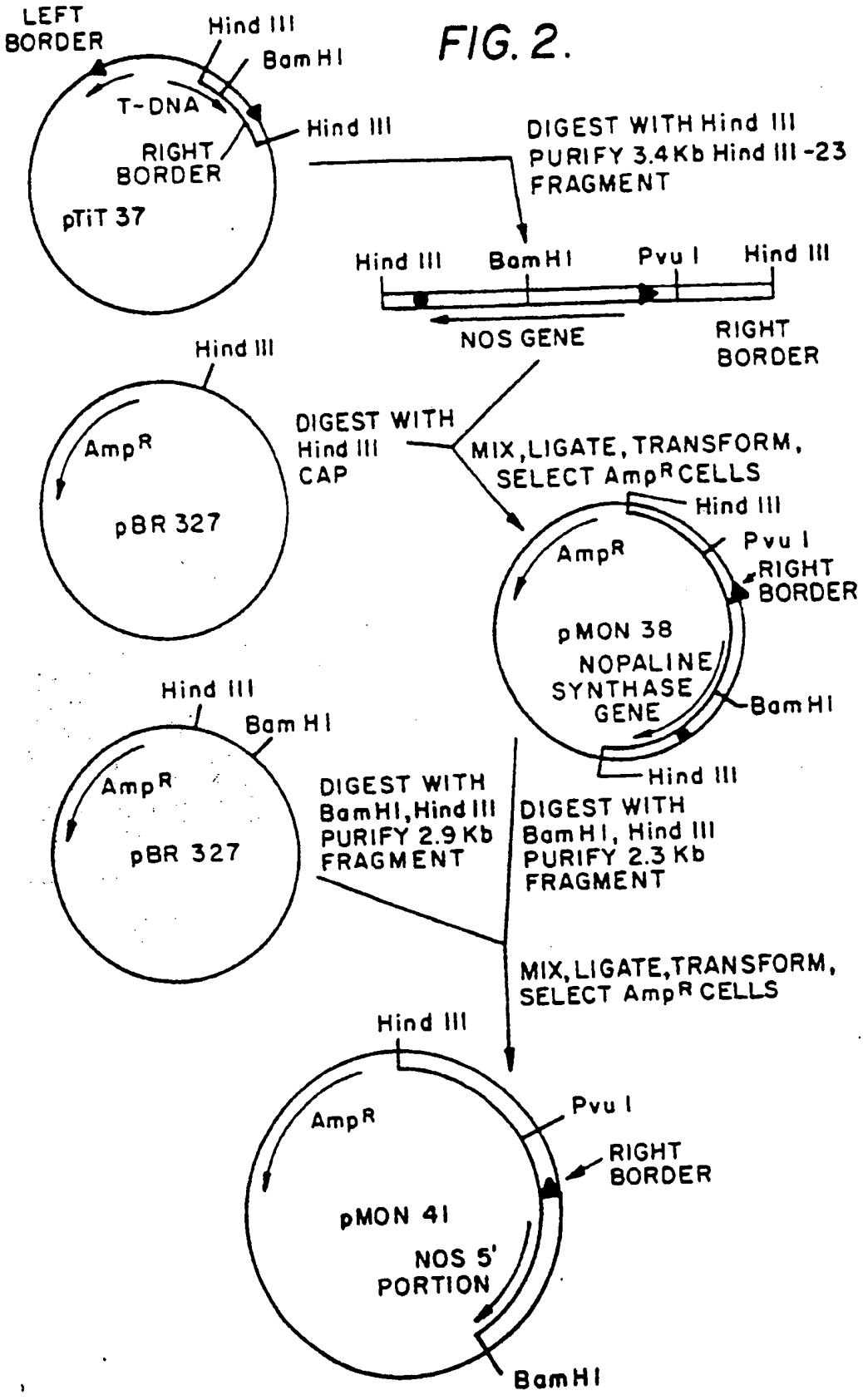

SELECT PLASMID HAVING T-DNA BORDER, A. TUM. MARKER, PLANT CELL MARKER, UNIQUE CLEAVAGE SITE, AND Ti HOMOLOGY
INSERT DESIRED GENE INTO PLASMID
INSERT PLASMID INTO A. TUM. CELL HAVING Ti PLASMID; CULTIVATE TO ALLOW CROSSOVER EVENT
SELECT A. TUM. CELLS HAVING CO-INTEGRATE PLASMIDS
CO-CULTIVATE A. TUM. CELLS WITH PLANT CELLS
INSERT CO-INTEGRATE PLASMIDS INTO PLANT CELLS
SELECT TRANSFORMED PLANT CELLS HAVING PLANT CELL MARKER
REGENERATE PLANTS FROM TRANSFORMED PLANT CELLS

FIG. 1.

FIG. 2.


LEFT BORDER
Hind III
Bam HI
T-DNA
RIGHT BORDER
Hind III
pTiT 37
DIGEST WITH Hind III
PURIFY 3.4Kb Hind III -23 FRAGMENT
Hind III
BamHI
Pvu I
Hind III
NOS GENE
RIGHT BORDER
Hind III
AmpR
pBR 327
DIGEST WITH Hind III CAP
MIX, LIGATE, TRANSFORM, SELECT AmpR CELLS
Hind III
Pvu I
AmpR
RIGHT BORDER
pMON 38
NOPALINE SYNTHASE GENE
BamHI
Hind III
Hind III
Bam HI
AmpR
pBR 327
DIGEST WITH BamHI, Hind III PURIFY 2.9Kb FRAGMENT
DIGEST WITH BamHI, Hind III PURIFY 2.3Kb FRAGMENT
MIX, LIGATE, TRANSFORM, SELECT AmpR CELLS
Hind III
Pvu I
AmpR
RIGHT BORDER
pMON 41
NOS 5' PORTION
BamHI

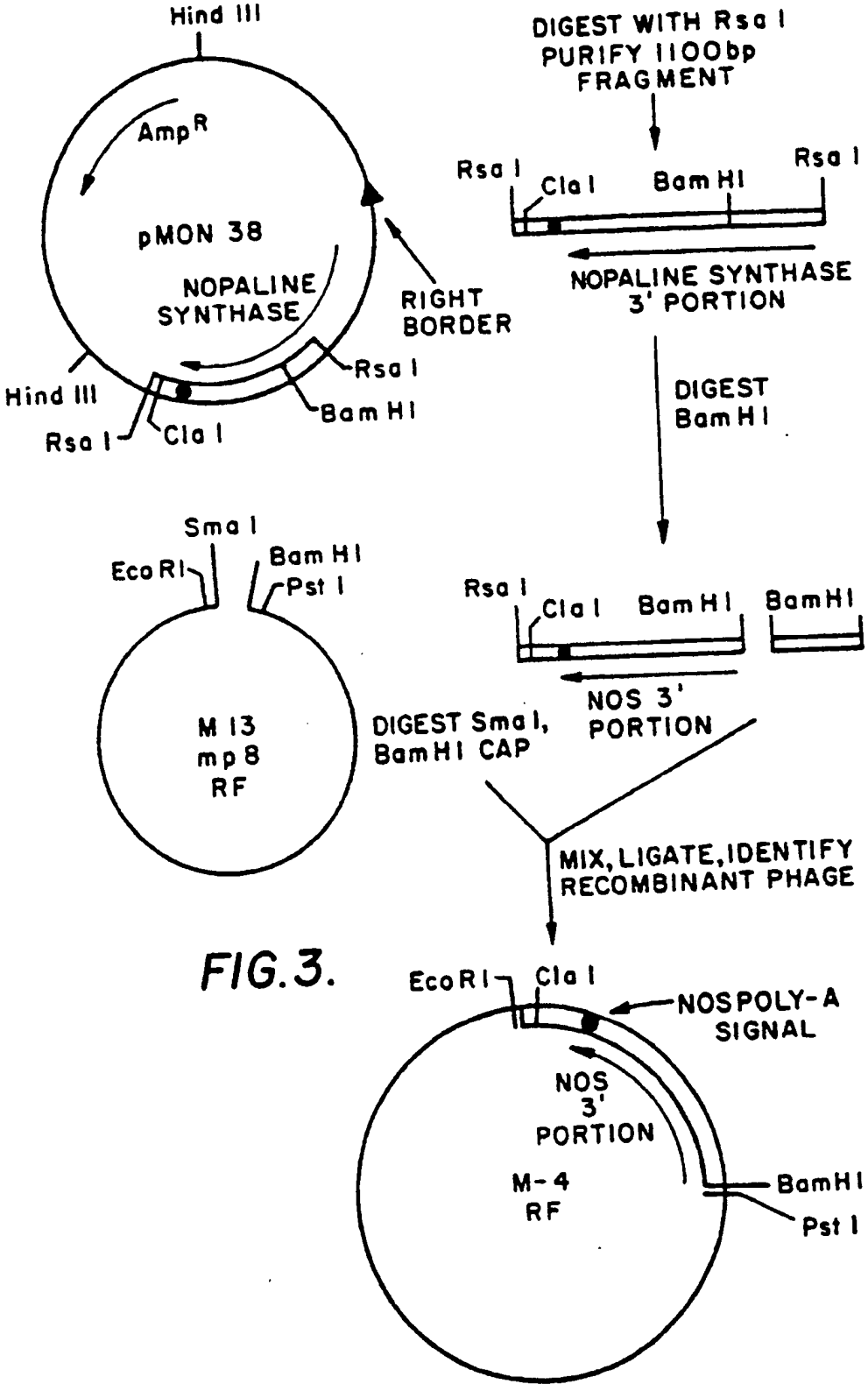

Hind III
Amp R
pMON 38
NOPALINE SYNTHASE
RIGHT BORDER
Rsa I
Bam HI
Hind III
Rsa I
Cla I
DIGEST WITH Rsa I
PURIFY 1100bp FRAGMENT
Rsa I
Cla I
Bam HI
Rsa I
NOPALINE SYNTHASE 3' PORTION
DIGEST Bam HI
Sma I
Eco RI
Bam HI
Pst I
M 13 mp 8 RF
Rsa I
Cla I
Bam HI
Bam HI
NOS 3' PORTION
DIGEST Sma I, Bam HI CAP
MIX, LIGATE, IDENTIFY RECOMBINANT PHAGE
Eco RI
Cla I
NOS POLY-A SIGNAL
NOS 3' PORTION
M-4 RF
Bam HI
Pst I

FIG. 3.

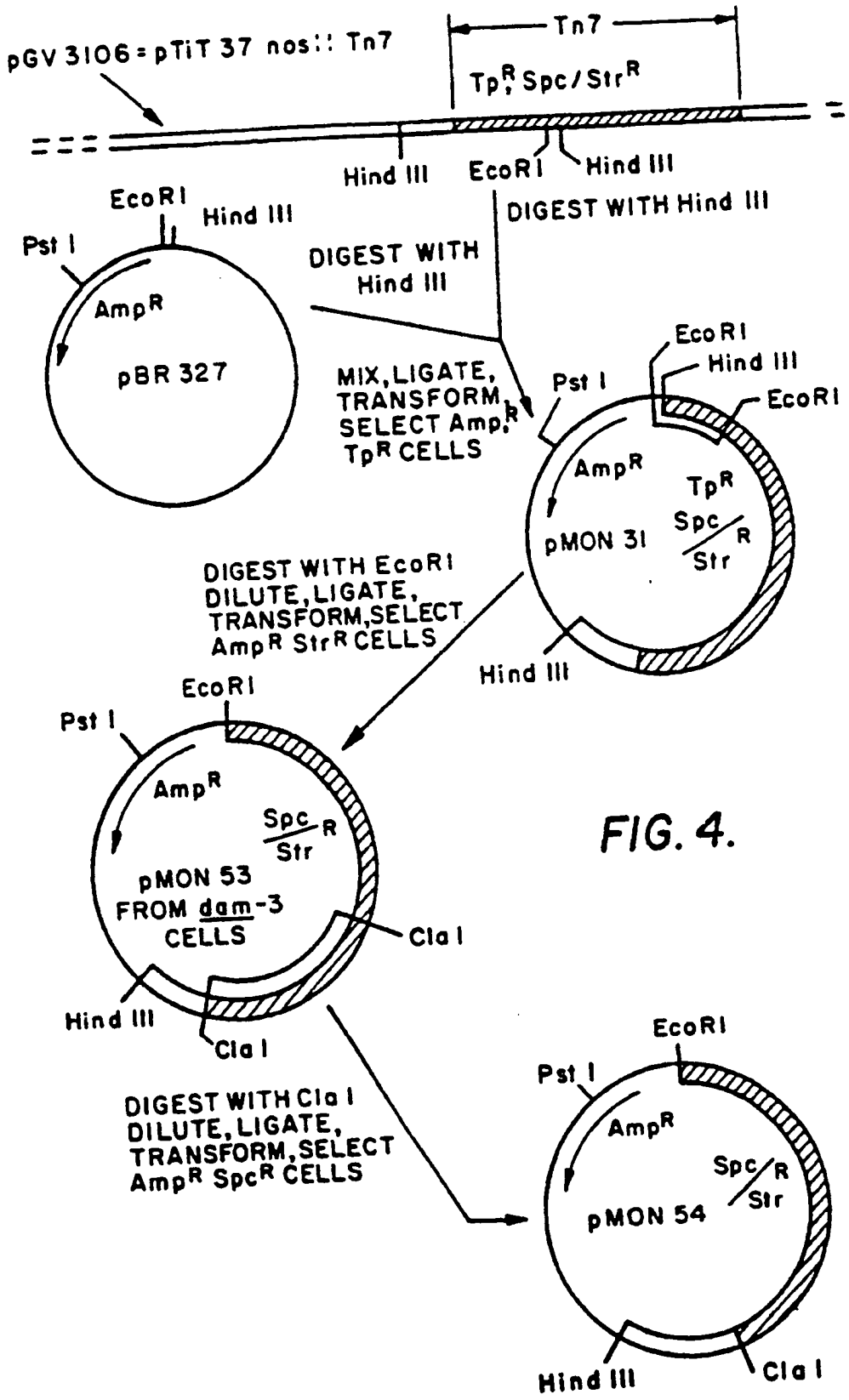

pGV 3106 = pTiT 37 nos::Tn7
Tn7
Tp^R Spc/Str^R
Hind III
EcoRI
Hind III
EcoRI
Hind III
Pst I
AmpR
pBR 327
DIGEST WITH Hind III
DIGEST WITH Hind III
MIX, LIGATE, TRANSFORM, SELECT Amp^R Tp^R CELLS
EcoRI
Hind III
Pst I
EcoRI
AmpR
TpR
pMON 31
Spc/Str R
Hind III
DIGEST WITH EcoRI
DILUTE, LIGATE, TRANSFORM, SELECT Amp^R Str^R CELLS
EcoRI
Pst I
AmpR
Spc/Str R
pMON 53
FROM dam-3 CELLS
Cla I
Hind III
Cla I
DIGEST WITH Cla I
DILUTE, LIGATE, TRANSFORM, SELECT Amp^R Spc^R CELLS
EcoRI
Pst I
AmpR
Spc/Str R
pMON 54
Hind III
Cla I

FIG. 4.

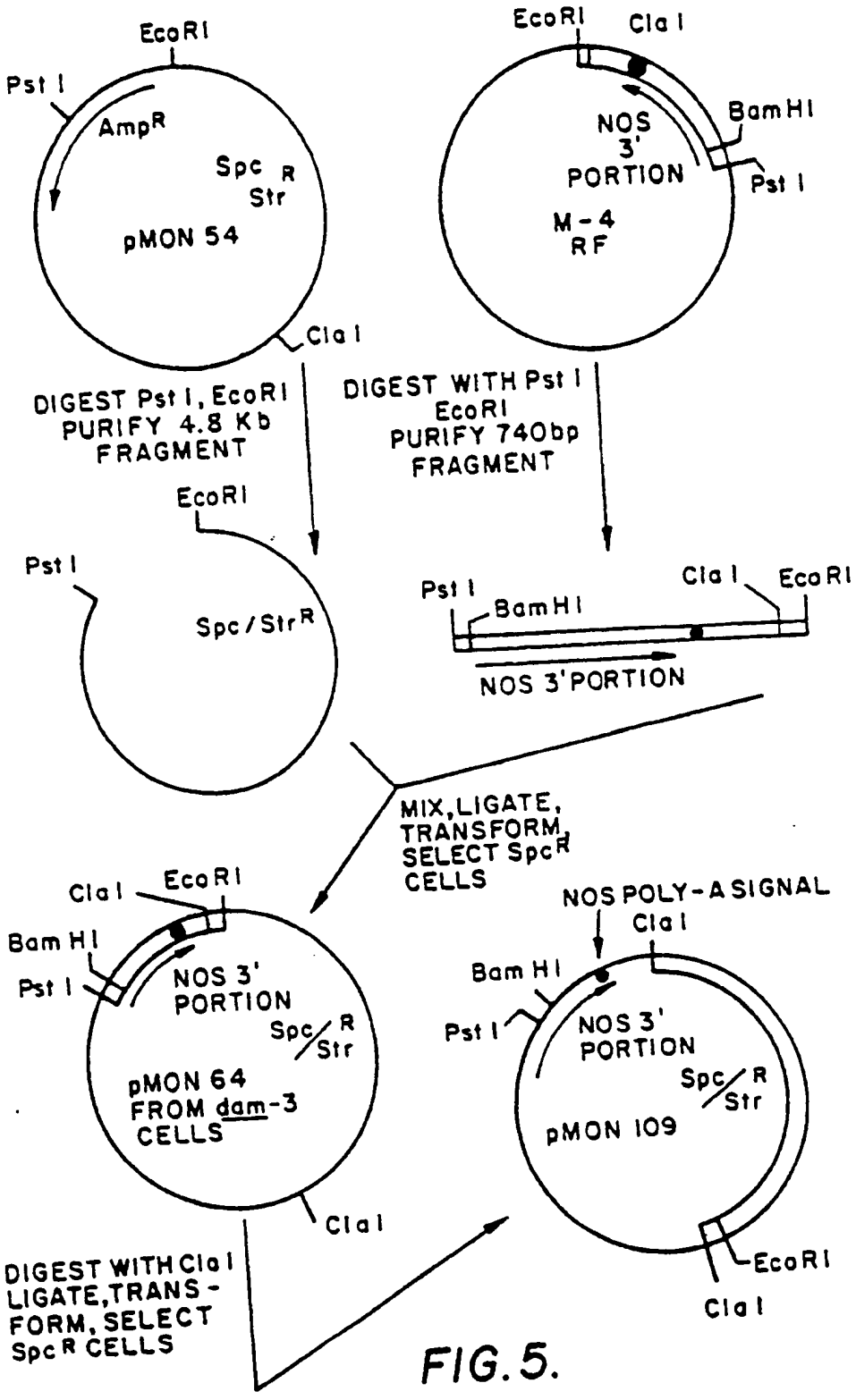

EcoRI
Pst I
AmpR
Spc R
Str
pMON 54
Cla I
EcoRI
Cla I
NOS
3'
PORTION
M-4
RF
BamHI
Pst I
DIGEST Pst I, EcoRI
PURIFY 4.8 Kb
FRAGMENT
DIGEST WITH Pst I
EcoRI
PURIFY 740bp
FRAGMENT
EcoRI
Pst I
Spc/Str R
Pst I
BamHI
Cla I
EcoRI
NOS 3'PORTION
MIX, LIGATE,
TRANSFORM,
SELECT SpcR
CELLS
Cla I
EcoRI
Bam HI
Pst I
NOS 3'
PORTION
Spc/Str R
pMON 64
FROM dam-3
CELLS
Cla I
NOS POLY-A SIGNAL
Cla I
Bam HI
Pst I
NOS 3'
PORTION
Spc/Str R
pMON 109
EcoRI
Cla I
DIGEST WITH Cla I
LIGATE, TRANS-
FORM, SELECT
SpcR CELLS

FIG. 5.

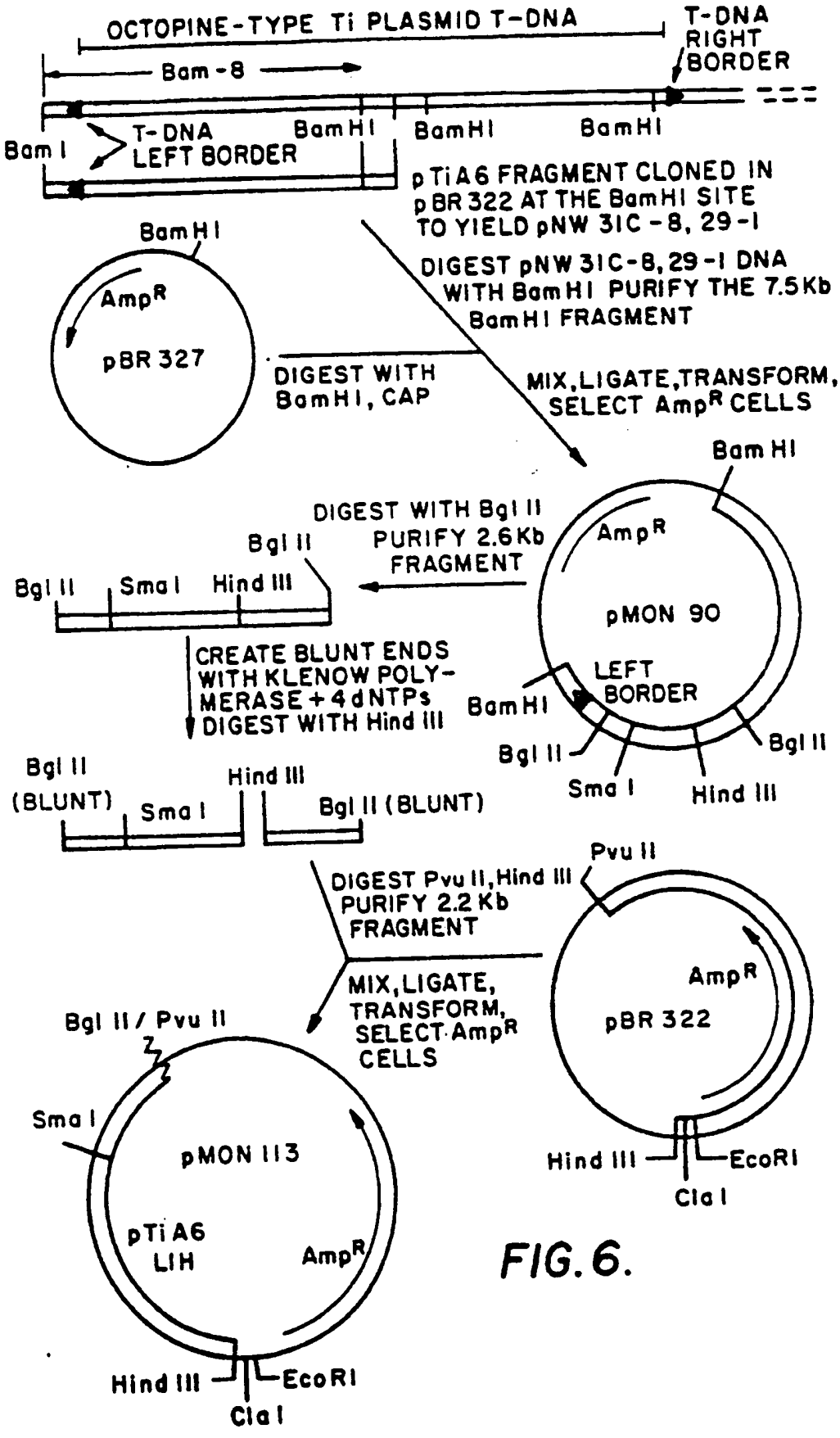

OCTOPINE-TYPE Ti PLASMID T-DNA
T-DNA RIGHT BORDER
Bam-8
Bam I
T-DNA LEFT BORDER
BamHI
BamHI
BamHI
pTiA6 FRAGMENT CLONED IN pBR322 AT THE BamHI SITE TO YIELD pNW 31C-8, 29-1
DIGEST pNW 31C-8, 29-1 DNA WITH BamHI PURIFY THE 7.5Kb BamHI FRAGMENT
BamHI
AmpR
pBR 327
DIGEST WITH BamHI, CAP
MIX, LIGATE, TRANSFORM, SELECT AmpR CELLS
Bam HI
AmpR
pMON 90
LEFT BORDER
BamHI
Bgl II
Sma I
Hind III
Bgl II
DIGEST WITH Bgl II
PURIFY 2.6Kb FRAGMENT
Bgl II
Sma I
Hind III
CREATE BLUNT ENDS WITH KLENOW POLYMERASE + 4 dNTPs
DIGEST WITH Hind III
Bgl II (BLUNT)
Sma I
Hind III
Bgl II (BLUNT)
Pvu II
DIGEST Pvu II, Hind III
PURIFY 2.2 Kb FRAGMENT
MIX, LIGATE, TRANSFORM, SELECT AmpR CELLS
AmpR
pBR 322
Hind III
EcoRI
Cla I
Bgl II / Pvu II
Sma I
pMON 113
pTiA6 LIH
AmpR
Hind III
EcoRI
Cla I

FIG. 6.

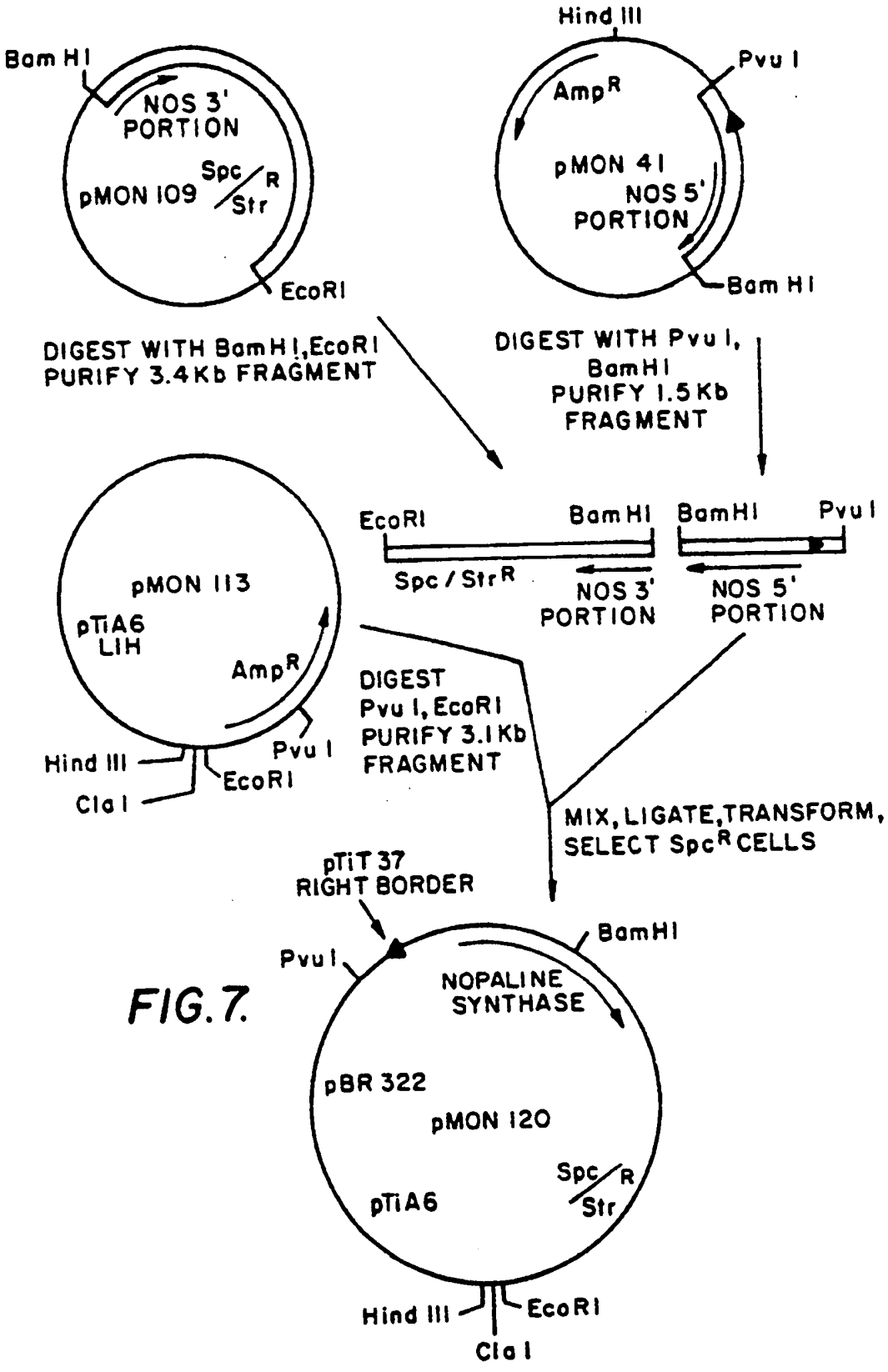
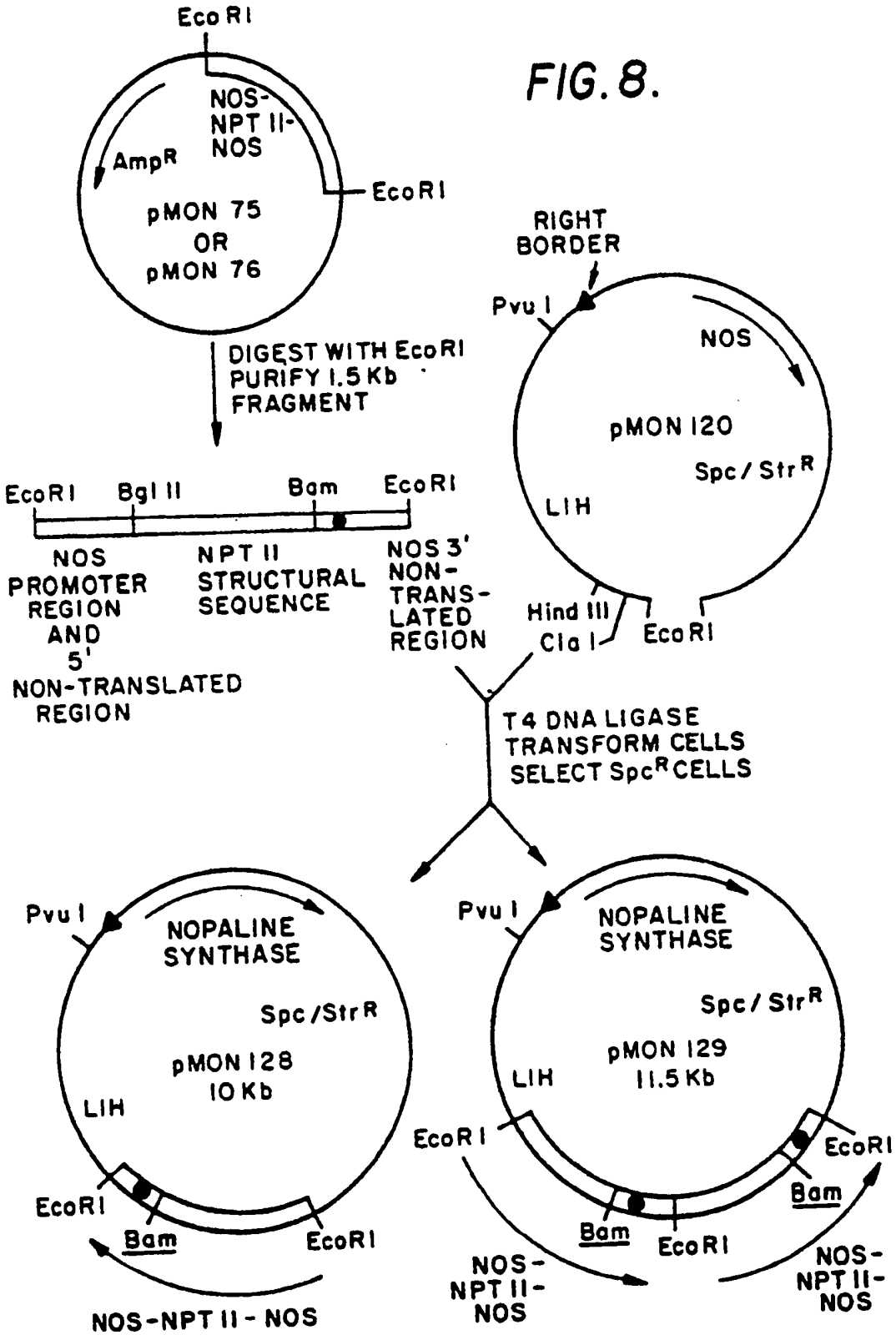

Bam HI
NOS 3' PORTION
pMON 109
Spc/Str R
EcoRI
Hind III
Pvu I
Amp R
pMON 41
NOS 5' PORTION
Bam HI
DIGEST WITH BamHI, EcoRI
PURIFY 3.4Kb FRAGMENT
DIGEST WITH Pvu I, BamHI
PURIFY 1.5Kb FRAGMENT
pMON 113
pTiA6 LIH
Amp R
Hind III
Cla I
EcoRI
Pvu I
EcoRI
BamHI
BamHI
Pvu I
Spc / Str R
NOS 3' PORTION
NOS 5' PORTION
DIGEST Pvu I, EcoRI
PURIFY 3.1Kb FRAGMENT
MIX, LIGATE, TRANSFORM, SELECT Spc R CELLS
pTiT 37 RIGHT BORDER
Pvu I
BamHI
NOPALINE SYNTHASE
pBR 322
pMON 120
pTiA6
Spc/Str R
Hind III
EcoRI
Cla I

FIG. 7.

FIG. 8.


Eco RI
NOS-
NPT II-
NOS
AmpR
EcoRI
pMON 75
OR
pMON 76
DIGEST WITH EcoRI
PURIFY 1.5 Kb
FRAGMENT
EcoRI
Bgl II
Bam
EcoRI
NOS
PROMOTER
REGION
AND
5'
NON-TRANSLATED
REGION
NPT II
STRUCTURAL
SEQUENCE
NOS 3'
NON-
TRANS-
LATED
REGION
RIGHT
BORDER
Pvu I
NOS
pMON 120
Spc / Str R
LIH
Hind III
Cla I
EcoRI
T4 DNA LIGASE
TRANSFORM CELLS
SELECT Spc R CELLS
Pvu I
NOPALINE
SYNTHASE
Spc/Str R
pMON 128
10 Kb
LIH
EcoRI
Bam
EcoRI
NOS-NPT II-NOS
Pvu I
NOPALINE
SYNTHASE
Spc/Str R
pMON 129
11.5 Kb
LIH
EcoRI
EcoRI
Bam
Bam
EcoRI
NOS-
NPT II-
NOS
NOS-
NPT II-
NOS

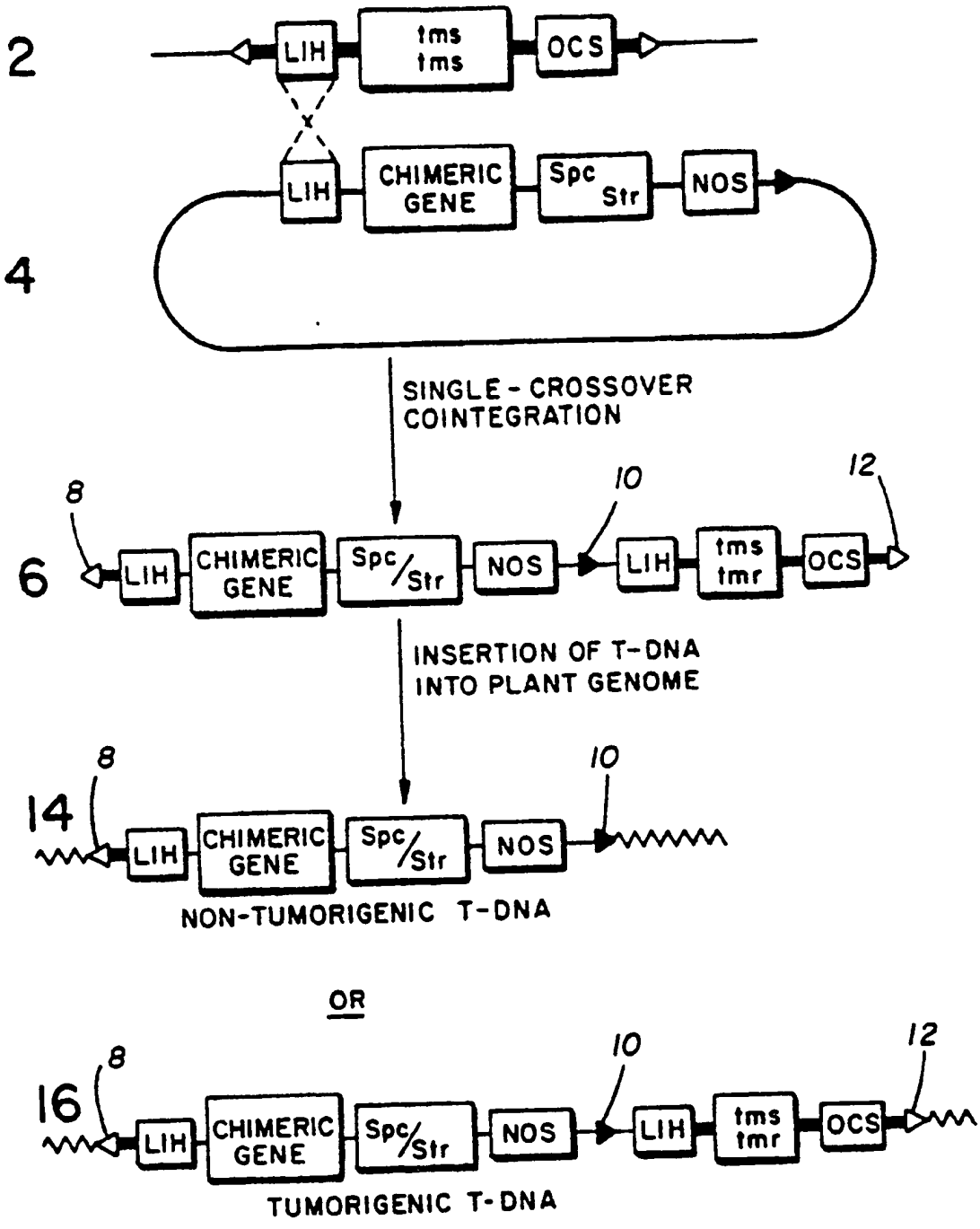

2
LIH
tms
tms
OCS
CHIMERIC GENE
Spc Str
NOS
4
SINGLE - CROSSOVER COINTEGRATION
8
10
12
6
LIH
CHIMERIC GENE
Spc/Str
NOS
LIH
tms
tmr
OCS
INSERTION OF T-DNA INTO PLANT GENOME
8
10
14
LIH
CHIMERIC GENE
Spc/Str
NOS
NON-TUMORIGENIC T-DNA
OR
8
10
12
16
LIH
CHIMERIC GENE
Spc/Str
NOS
LIH
tms
tmr
OCS
TUMORIGENIC T-DNA

FIG. 9.

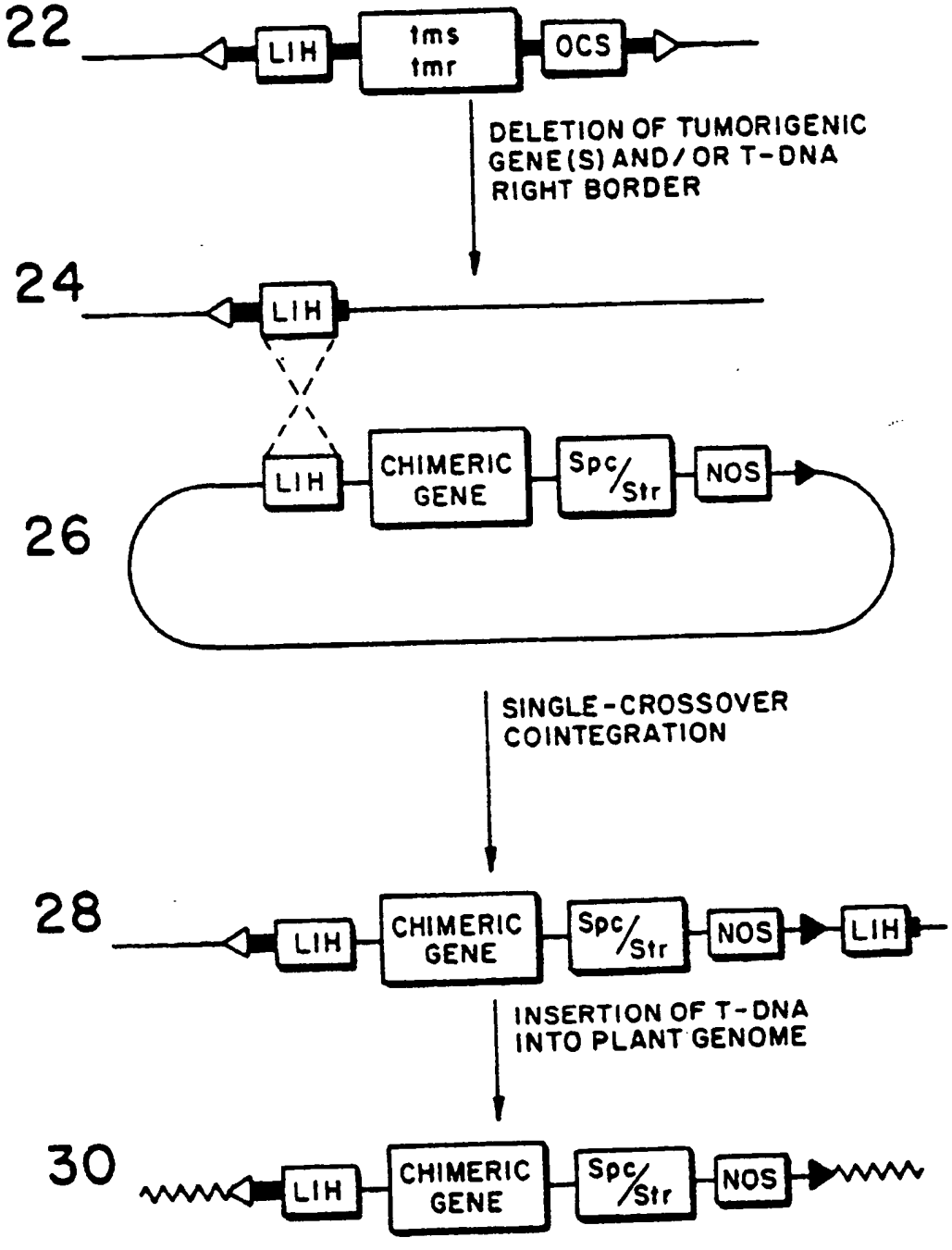

22
LIH
tms
tmr
OCS
DELETION OF TUMORIGENIC GENE(S) AND/OR T-DNA RIGHT BORDER
24
LIH
LIH
CHIMERIC GENE
Spc/Str
NOS
26
SINGLE-CROSSOVER COINTEGRATION
28
LIH
CHIMERIC GENE
Spc/Str
NOS
LIH
INSERTION OF T-DNA INTO PLANT GENOME
30
LIH
CHIMERIC GENE
Spc/Str
NOS

FIG.10.

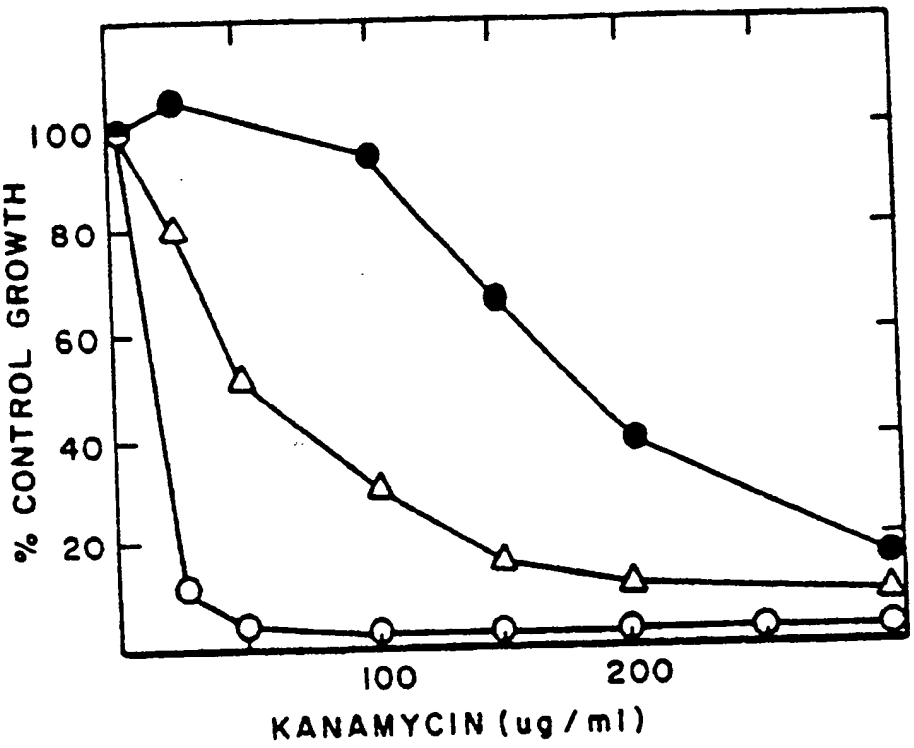


FIG. 11.